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Analysis of Soil and Groundwater Microbial Population Dynamics at in situ Bioremediation Sites in California and Texas

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ANALYSIS OF SOIL AND GROUNDWATER MICROBIAL POPULATION DYNAMICS AT *IN SITU* BIOREMEDIATION SITES IN CALIFORNIA AND TEXAS

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ABSTRACT

Traditional methods for assessing *in situ* microbial communities often provide limited information on substrate utilization in bioremediation processes. The goal of this research was to assess new methods for describing the microbial communities found in the groundwater and affected contaminated soils documenting changes in community structure, population dynamics, substrate utilization and biodegradation of constituents of concern (CoC) during a site remediation. Two *in situ* bioremediation pilot studies using biological plugs are being conducted, one in the California San Francisco Bay Area (Sunnyvale) and one in the East Texas Piney Woods (Longhorn). The hypothesis is that changes in community structure, population dynamics, substrate utilization and biodegradation of CoC will be successfully described by EcoPlate™ and MicroPlate™ (Biolog, LLC) assays, population counts, and MicroTox® acute toxicity tests. Chlorinated aliphatic hydrocarbon and heterotroph plate counts were used to find the number of colony-forming units. The biological plugs successfully introduced chlorinated aliphatic degrading microorganisms to affected areas. At the Sunnyvale site, the average colony forming unit (CFU)/ml tracked reductions in CoC from August 2012 through January 2014. Reductions in CoC, in particular, trichloroethylene (TCE), were seen over a defined time frame of months to years for Sunnyvale. There was a correlation among increased microbial species diversity and abundance and decreased CoC. The average well color development (AWCD) maximum value increased almost 10 fold, the functional diversity and Shannon-Weaver index values showed notable improvement, and the carbon substrate utilization did not show major changes across all monitoring wells on site. At the Longhorn site, the average CFU/ml varied in population totals providing no clear indication of CoC reduction. Plate counts decreased

initially but then started to rise again. The number of samples with a high functional diversity and AWCD also increased. Unfortunately, more samples showed a zero for the Shannon-Weaver index. Carbon substrate utilization showed modest improvement. Since the Longhorn site has been in operation for only 1.5 years compared to Sunnyvale's 5 years, a clearer picture of Sunnyvale's improvement may be seen. Assays provided correlated assessment of community viability and functionality for both sites.

1 INTRODUCTION

1.1 Research Purpose

Two bioremediation sites are being studied for the effectiveness of *in situ* biological plugs to remove unwanted chlorinated aliphatic hydrocarbons: a California San Francisco Bay Area site (herein referred to as Sunnyvale) and an East Texas Piney Woods site (herein referred to as Longhorn). The goal of this research was to assess new methods for describing the microbial communities found in the groundwater and affected contaminated soils so as to document changes in community structure, population dynamics, substrate utilization and biodegradation of constituents of concern during a site remediation. The hypothesis is that changes in community structure, population dynamics, substrate utilization and biodegradation of constituents of concern will be successfully described by Biolog EcoPlates™ and MicroPlates™, population counts, and MicroTox® acute toxicity tests.

Benefits of remediation include "protection of human health, prevention of further damage to natural resources and the environment, safeguarding of valuable water resources, such as aquifers and rivers, reuse of derelict land, preventing unnecessary development of green-field sites, increased land values, and assuagement of public concern" [1]. The advantage of biological treatment is that the hazardous material is chemically altered by biological agents to nontoxic byproducts such as carbon dioxide, water and biomass thus eliminating the need for disposal [2].

In aerobic bioremediation, "the contaminant degrading potential of these microorganisms is enhanced through the addition of essential reactants (inorganic and

organic nutrients, water, and oxygen)" [3]. The aerobic heterotrophs produce enzymes that oxidize "substrates by transfer of electrons to molecular oxygen" [3].

The aerobic bioremediation technology used in this research is immobilized microbe bioreactors, or bio plugs. They are innovative, efficient, economical, low-intervention, and in-situ [4]. They are used "for both saturated and unsaturated contaminated soils" and "designed to rapidly increase the oxidation of organic contaminants by placing an adapted consortia of microorganisms in close proximity to the contaminants of concern" [4].

2 REVIEW OF LITERATURE

2.1 Chemicals of Concern

Various volatile organic compounds (VOCs) and metals are found in the groundwater at these two sites. The history of each site is covered in sections 2.2.1 and 2.2.2. At Sunnyvale, the constituents of concern are 1,1,1-trichloroethane (1,1,1-TCA), trichloroethylene (TCE), tetrachloroethylene (PCE), 1,1-dichloroethylene (1,1-DCE), cis-1,2-dichloroethylene (cis-1,2-DCE), trans-1,2-dichloroethylene (trans1,2-DCE), 1,1,2-trichloro-1,2,2-trifluoroethane (CFC-113), trichlorofloromethane (TCFM), hexavalent chromium and nitrate [5]. At Longhorn, the constituents of concern are 1,1-DCE, PCE, TCE, antimony, thallium and vinyl chloride [6]. Our research focuses on the main constituent of concern, TCE. The EPA's maximum contaminant level (MCL) for TCE is 5 ppb [7]. California has an additional public health goal (PHG) of 1.7 ppb for TCE [8].

2.1.1 Trichloroethylene

2.1.1.1 Synthesis, Source & Use

Trichloroethylene (TCE) "is a nonflammable, colorless liquid at room temperature with a somewhat sweet odor and a sweet, burning taste" [9]. It is only manufactured in Freeport, TX at DOW Chemical and in Lake Charles, LA at PPG Industries [10]. At PPG Industries, a single step oxychlorination process takes ethylene dichloride and reacts it "with chlorine and/or hydrogen chloride and oxygen to form the trichloroethylene and tetrachloroethylene" [9]. DOW Chemical uses a direct chlorination process where "ethylene dichloride is reacted with chlorine to form trichloroethylene and tetrachloroethylene" [9]. In 2011, they produced 270 million pounds combined [10]. TCE is disposed of via "incineration after mixing with a combustible fuel" [9]. It must be

completely combusted or else phosgene can be formed [9].

In 2004, in the U.S., 73% of TCE was used as a hydrofluorocarbon (HFC-134a) intermediate, 24% was used in metal degreasing, and 3% was used miscellaneously [10], which can include uses "by the textile processing industry to scour cotton, wool, and other fabrics" [9]. It can also be found in household products such as paint strippers, adhesives, and spot removers [9]. Its now banned uses include "general and obstetrical anesthetic; grain fumigant; skin, wound, and surgical disinfectant; pet food additive; and extractant of spice oleoresins in food and of caffeine for the production of decaffeinated coffee" [9]. Abrahamsson et al. discovered that TCE is produced "by several species of marine macroalgae and at least one species of marine [red] microalgae" [9].

2.1.1.2 Natural Concentrations

TCE is almost ubiquitous. It has been found in at least 861 National Priorities List (NPL) sites [9]. TCE is a hazardous air pollutant (HAP) under the Clean Air Act (CAA) and has a reportable quantity limit of 100 pounds [10]. It is emitted into the atmosphere at a rate of 7.6 Gg/year [10]. The upper limit for the remote background concentration of TCE in North America in the air is 0.02 $\mu g/m^3$ [10]. Assuming an air concentration range of 100-500 ppt TCE and a water concentration range of 2-7 ppb TCE, "the average daily air intake of trichloroethylene can be estimated at 11-33 μg/day" and "the average daily water intake of trichloroethylene can be estimated at 2-20 μg/day" [9]. TCE degrades at higher temperatures, so there is a higher level of TCE in the atmosphere in the winter months [9]. Most people can smell TCE in the air when it is around 100 ppm [9]. In 2005, 4.9% of samples taken from groundwater supplied public water systems (PWS) and 14.8% of samples taken from surface water supplied by PWS contained TCE according

to EPA monitoring [10]. From 1996-2000, 30 table-ready food items from supermarkets in the U.S. contained TCE [10]. Fortunately, all blood samples taken from 2,150 people in the U.S. from 2001 to 2004 had TCE concentrations below the detection limit of 0.012 ng/mL [10].

2.1.1.3 Exposure Limits

Because TCE is toxic the Occupational Safety and Health Administration (OSHA), has set enforceable permissible exposure limits (PELs) for TCE of "100 ppm for an 8-hour time weighted average [TWA], [and] 200 ppm ceiling concentration for an 8-hour shift, or a maximum peak of 300 ppm during not more than 5 minutes in any 2 hours of an 8 hour shift" [10]. There are also several recommended exposure guidelines that describe risk. EPA's Chronic Reference Dose (RfD) is the maximum acceptable oral dose. It is 0.0005 mg/kg/day. The Chronic Reference Concentration (RfC) is the maximum acceptable inhalation concentration. It is 0.0004 ppm. These are adopted as chronic MRLs (minimal risk levels) [10]. The Department of Energy's temporary emergency exposure limits (DOE-TEEL) at level 0 is 10 ppm [10]. This is the threshold concentration below which most people have no adverse health effects for 1 hour of exposure. The acute exposure guideline levels (AEGL) at various lengths of exposure and concentrations are described in Table 1 [10].

Table 1. Acute Exposure Guideline Levels [10]

Level	Exposure Time	Concentration (ppm)
Level 1	10 min	260
	30 min	180
	60 min	130
	4 hours	84
	8 hours	77
Level 2	10 min	960
	30 min	620

(Table 1 continued)

Level	Exposure Time	Concentration (ppm)
	60 min	450
	4 hours	270
	8 hours	240
Level 3	10 min	6100
	30 min	6100
	60 min	3800
	4 hours	1500
	8 hours	970

Level 1 is the airborne concentration above which the population, including the susceptible, experience symptoms such as discomfort and irritation which are not disabling and are reversible. Level 2 is irreversible, has long-lasting effects, and one may not be able to escape the hazardous area. Level 3 has life-threatening health effects or death [10]. While no human LC50 (concentration that will kill half the population) values have been reported, "an LC50 value for acute exposure in rats was reported as 12,500 ppm for a 4-hour exposure" [9].

The carcinogenicity of TCE varies depending on who is asked. According to the EPA, TCE is "'carcinogenic to humans' by all routes of exposure" [10], and according to the International Agency for Research on Cancer (IARC), TCE is a group 1 carcinogen meaning it is "carcinogenic to humans" [10]. But, according to the National Toxicology Program (NTP) of the HHS, TCE is "reasonably anticipated to be a human carcinogen" [10], and according to the American Conference of Governmental Industrial Hygienists (ACGIH), TCE is an A2 carcinogen meaning it is a "suspected human carcinogen" [10].

2.1.1.4 Susceptibility

TCE is a toxic chemical. Three studies found that TCE reacted with other substances to have synergistic effects. In the first, co-exposure to mercury increased TCE

induced autoimmune hepatitis in autoimmune prone MRL+/+ [Murphy Roths Large] mice [10]. It also generated a "liver-specific antibody response" [10]. In the second, mice exposed to TCE vapors at 3,000 ppm and noise at 95 dB had significantly greater hearing loss at 4kHz than exposure to each independently [10]. In the third, exposure to ethanol and TCE increased the concentration of TCE in blood and breath, because both compete for enzymatic sites causing TCE metabolism to decrease [10].

Infants and children are more susceptible than adults to TCE toxicity [10]. TCE is "absorbed in greater amounts in children exposed by inhalation due to increased ventilation rates per kg body weight and the fact that alveolar surface area is 2-fold higher in infants compared to adults" [10]. Children also drink more water per kilogram body weight. Infants can be exposed to TCE via breast milk. Because infants and children "have a higher concentration of lipophilic compounds in their fat," they have a higher concentration of TCE in their fat [10]. Higher metabolic rates will also lead to greater toxicity [10]. Adults with human herpesvirus 6 are more likely to have TCE-induced skin disorders and hepatic dysfunction [10]. Also, males are more susceptible to TCE-induced renal toxicity than females [10]. There can also be gene-related susceptibility. People with an allele for human leucocyte antigen (HLA-B*1301) may experience hypersensitivity dermatitis [10].

2.1.1.5 Health Effects of Exposure

There are a plethora of effects seen from TCE exposure, but "there is no clinical disease state that is unique to trichloroethylene exposure" [9]. Death has been tied with inhaling high concentrations of TCE [10]. Some effects seen in rats and humans after varying amounts of exposure are: asthma; bronchiolitis and alveolitis; ischemic heart

disease; muscle necrosis; jaundice; hepatomegaly; hepatosplenomegaly; hepatitis; liver failure; changes in urinary proteins causing renal toxicity; perivascular interstitial inflammation; glomerulonephritis; decreased levels of follicle-stimulating hormone (FSH), testosterone, and sex-hormone binding globulin; increased dehydroepiandrosterone sulfate (DHEAS); eosinophilic fasciitis; hypersensitivity dermatitis and other skin disorders; Stevens-Johnson syndrome; ocular irritation; scleroderma (systemic sclerosis); decreased levels of lymphocytes, T cells, CD4+ T cells, CD8+ T cells, and natural killer (NK) cells; unconsciousness; amnesia; polyneuropathy; myoclonic encephalopathy; hearing loss; hyperzoospermia; abnormal sperm morphology; testicular atrophy; decreases in sperm count and motility; increased risk of congenital heart defects; increased risk for kidney cancer [10]; ventricular fibrillation; and central nervous system depression [9].

Likewise, effects resulting from oral exposure include: hepatorenal failure and death; pulmonary congestion and edema; cardiac arrhythmia; sinus tachycardia; vomiting; diarrhea; hemorrhagic gastritis; abdominal perforation and necrosis; skeletal muscle damage; liver ailments; immunosuppression; accelerated autoimmune response; liver, pancreas, lung and kidney inflammation; hearing impairment; tremor; motor restlessness; coma; decreased dopamine levels; and verbal naming/language impairment in children [10].

2.1.1.6 Mechanism of Action

After TCE enters the body, it is rapidly distributed to tissues because it quickly crosses membranes. See Figure 1. TCE is stored in adipose tissue with a half-life of 3.5-5 hours [9]. The target organs are the nervous system, liver, heart, kidneys, immune

system, male reproductive system, and the developing fetus [10]. The nervous system is most sensitive to acute toxicity from inhaling TCE [9]. It is metabolized via P450 monooxidase and glutathione pathways [9]. It is unknown why one path may be preferred over the other, but they are in competition [11]. The metabolites are primarily the toxic compounds. For example, "the CNS-depressant effects of TCE are due in part to the sedative properties of the metabolites trichloroethanol (TCOH) and chloroform" [12]. Metabolism occurs in the respiratory tract, liver and kidneys [10]. There is also evidence that TCE is metabolized in the male reproductive tract in the epididymal epithelium and Leydig cells "by CYP2E1 to chloral, trichloroethanol, and TCA" [10]. TCE is metabolized via GSH (glutathione) conjugation and P450 oxidation producing reactive electrophiles such as TCE oxide [11].

Oxidative metabolism occurs in the respiratory tract [10]. TCE is oxidized to chloral and eliminated [10]. After inhalation exposure of 1 ppm for 6 hours, the half-life of TCE is 14-23 hours in alveolar air [10]. 37-64% of inhaled TCE is absorbed in the lungs [9]. 40-75% of the retained inhaled dose of TCE is metabolized [9]. One phenomenon is that TCE can be excreted in the breath after dermal exposure [10].

Oxidative and conjugative metabolism occurs in the liver. TCE produces trichloroacetic acid (TCA), trichloroethanol-glucuronide conjugate (DCVG or S-1,2-dichlorovinyl-glutathione), and dichloroacetic acid (DCA) metabolites, "which are excreted in urine" [10]. TCE is first converted to chloral and then to TCA and trichloroethanol [10]. TCA can be converted to DCA [10]. Trichloroethanol is converted to TCA, DCVG, or DCA. DCA can be metabolized to monochloroacetic acid or glyoxylic acid, forming oxalic acid and carbon dioxide [10]. DCVG is secreted in the bile

to the gastrointestinal tract, “from where it can be reabsorbed as trichloroethanol (first order) representing enterohepatic circulation” [10]. TCE is metabolized to chloral hydrate and TCE oxide by cytochrome P450 2E1 [11], where “the chloral hydrate is [then] oxidized by chloral hydrate dehydrogenase to TCA or reduced by alcohol dehydrogenase to trichloroethanol” [9]. Chloral hydrate is mutagenic [10]. TCE oxide is rearranged into acyl chlorides, which react with lysine to create N^6-formyl-lysine and N^6-dichloroacetyl-lysine protein adducts and unstable esters [11]. TCE oxide irreversibly inactivates P450 2E1, P450 2B1, and NADPH-P450 reductase [11]. TCE oxide can be converted to dichloroacetyl chloride, which can be converted to DCA [10].

Glutathione conjugation occurs in the kidneys, too. TCE is converted to DCVG, which is then converted to DCVC [S-1,2-dichlorovinyl-L-cysteine], which is then activated to a cytotoxic product or eliminated by conversion to N-acetyl-S-dichlorovinyl-L-cysteine (NAcDCVC) [10]. The halftime for renal elimination of trichloroethanol and trichloroethanol glucuronide is about 10 hours following TCE exposure [9]. “The urinary excretion of TCA is much slower” and its halftime is about 52 hours because it “is very tightly and extensively bound to plasma proteins” [9].

The biomarkers to identify exposure are DCVG in blood, an increased glutathione-S-activity in liver and kidneys, increased urinary α1-microblobulin, and increased glutathione transferase alpha in urine [10]. The problem is that most of these biomarkers are not unique to TCE exposure [10]. There are some aspects of the mechanism of TCE metabolism is still unknown or speculated.

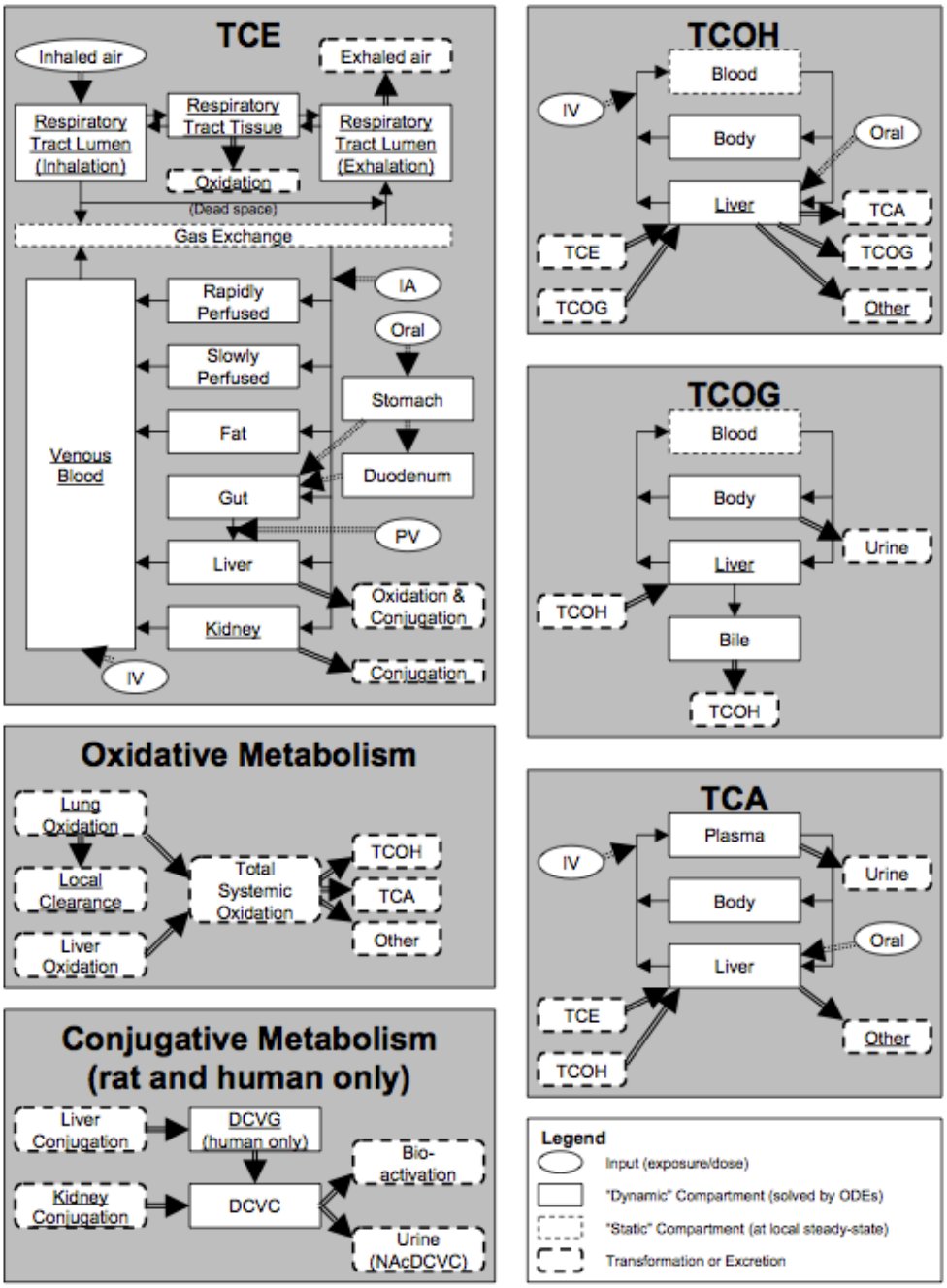


Figure 1. Metabolic Routes of TCE [10]

2.2 Bioremediation Technology

Bio plugs are used to inoculate the groundwater with the needed microorganisms to degrade the constituents of concern. Bio plugs are "an in situ technology that uses preselected acclimated microorganisms permanently immobilized or attached to a porous

matrix and inserted into soils/sediments as a seeding device for both saturated and unsaturated zones" [3]. The bio plug is constructed using "slotted PVC or HDPE pipe containing an inert porous material that serves as a structural support matrix for the microbial consortia" [4]. The consortia of microorganisms are "selected for their abilities to biodegrade the contaminants of concern, as well as their stability, viability after storage, nonpathogenicity, and ability to compete with indigenous microorganisms" [4]. If the indigenous microorganisms have adapted to site-specific contaminants, they can also be isolated and fixed to the immobilized bed [4]. Bio plugs are inserted vertically into the contaminated zone(s) as seen in Figure 2.

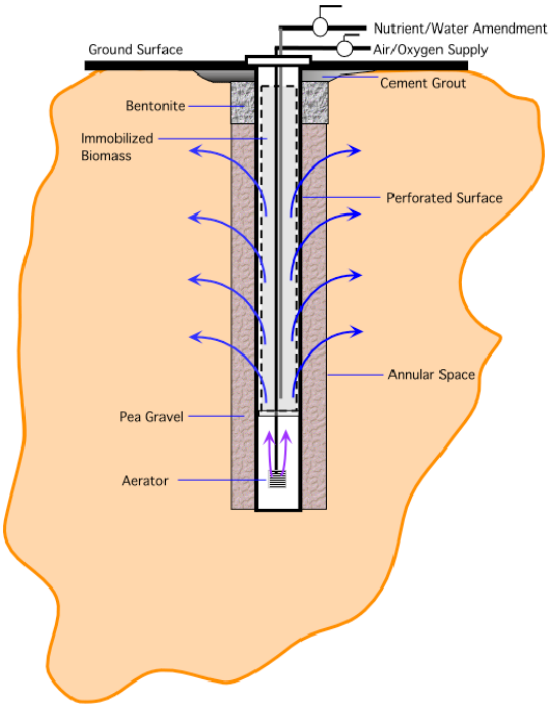


Figure 2. *In situ* Biological Plug [4]

Air is fed into the bio plug, which keeps the bio plug aerobic and extends the oxygen rich water into the surrounding area. Nutrient/water amendment is pumped through the bio plugs as a carbon nutrient source to maintain microbial activity [3].

Groundwater flow through the immobilized bed facilitates the mineralization of organic compounds [4]. Excess growth of microbes cause cells to bleed off, exit the bio plug, and "percolate through the unsaturated zone" [4]. In summary, the bio plug is a mini in situ biological reactor growing and delivering high concentrations of contaminant specific microbes into the contaminated zone. The radius of impact for each bio plug depends on host matrix, hydraulic conductivity, percentage of water saturation, and air and water flow rates [4]. For bio plug installation instructions, see Appendix C. Site specific bio plug information is described below.

2.2.1 Lockheed Martin Space Systems Company: Sunnyvale Site Plan

On site photos were not allowed, so photos were taken from the street (Figures 3 and 4).

Figure 3. Entrance of Lockheed Martin and Buildings as Seen from the Street

Figure 4. Yahoo! Building Located Downstream of Contaminated Groundwater

The Sunnyvale site builds submarine missiles. Buildings 181 and 182 are sources of the VOC contamination [13]. There are five units in these buildings, which were built in the late 1950s [13]. In building 181, there is a former machine shop and testing pit [13]. In building 182, there is a former beryllium shop, a former 440-gallon vapor degreaser, a paint spray/paint mixing rooms, and a sanitary sewer [13]. The sanitary sewer line north of the buildings is the probable source [13]. The VOCs are present in the First, Second, 2B and Intermediate Transmissive Zones (TZs), with the highest concentration in the Intermediate Zone (IZ) [13]. The VOCs are Dense Non-Aqueous Phase Liquids (DNAPL). The extent of the VOCs in the IZ is 150 feet downgradient (north) of the sewer line [13]. The vertical extent is probably 90 feet below ground surface [13]. 59 bio plugs were installed with each having a radius of influence of 12 to 15 feet (Figure 5). Soil samples were taken between 2002 and 2005, and "TCE was detected in 24 of 61 samples at concentrations ranging from 5 to 710,000 µg/kg" [13].

The highest concentrations were from samples collected at depths of 70 feet bgs or greater [13]. Groundwater samples were taken during the same period, and TCE was detected in five of six First Transmissive Zone samples at concentrations ranging from 1.7 to 63 µg/L, eight Second Transmissive Zone samples at concentrations ranging from 5.3 to 120,000 µg/L, 14 of 23 2B Transmissive Zone samples at concentrations ranging from 1.2 to 91,000 µg/L, and 11 of 36 Intermediate Zone samples at concentrations ranging from 0.9 to 230,000 µg/L [13].

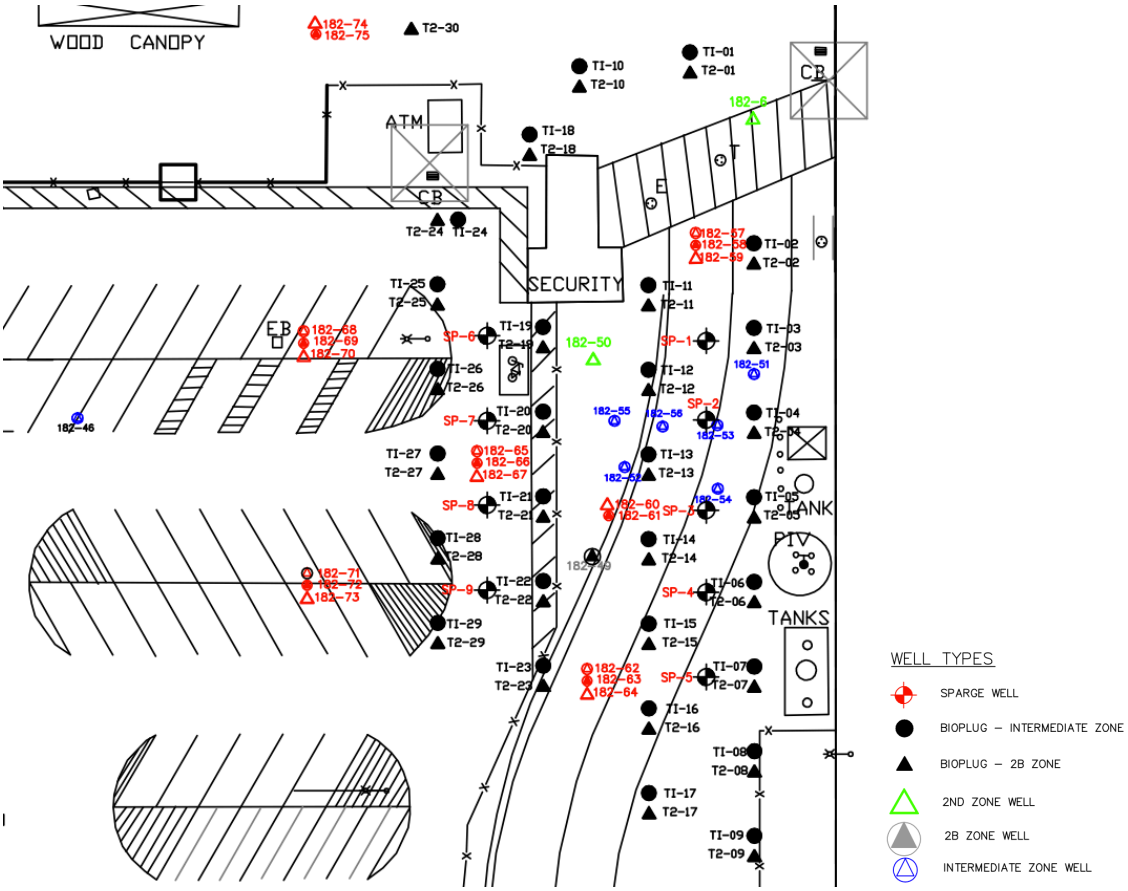


Figure 5. Sunnyvale Bioremediation Site Plan [13]

2.2.2 Longhorn Army Ammunition Plant Site Plan

Longhorn site 35B(37) was a chemical laboratory built in the 1950's. It "encompasses approximately 12.2 acres and is located in the north-central portion of Longhorn north of the intersection of Avenue P and 18th Street" [6]. The area is now a

"mixture of wooded and grassy vegetation-covered areas" [6]. Longhorn "was placed on the Superfund National Priorities List (NPL) on August 9, 1990" [6]. The plant "operated until 1997 when it was placed on inactive status" [6]. The plume size is about 105,000 square feet [6]. The CoC are primarily in the upper shallow zone [6]. The In Situ Microbial Bioreactor Enhanced Bioremediation Technology is part of a three-year pilot project. It is currently estimated to take 20-39 years to clean up the area under monitored natural attenuation [6]. Implementing the pilot project will hopefully achieve a shorter remediation time [6]. The pilot project includes "installation of the bio plug treatment system, installation of additional monitoring wells, process and performance monitoring during system operation, management of investigative derived waste, removing and plugging the bio plug bore holes at the end of the test period, plugging and abandonment of monitoring wells not designated for long-term monitoring, and reporting" [6]. The surface runoff flows northwest into Goose Prairie Creek, which drains into Caddo Lake, a drinking water source [6]. The top-confining layer "ranges in thickness from 7.5 to 18 feet" [6]. It "consists of the Quaternary silty clay underlain by alternating layers of clayey sand, silty sand, and poorly sorted sand of the Wilcox Group" [6]. Groundwater is 16 to 19.3 feet below ground surface in the upper shallow zone, 47 feet in the lower shallow zone, and 70 feet in the intermediate zone [6]. The groundwater flow is south-southeast at an average groundwater flow rate of 0.0496 feet/day [6]. The high VOC concentrations are located in the upper shallow groundwater zone [6]. The center of mass of the TCE plume is near monitoring well WW08, while the center of mass of the PCE plume is near monitoring well WW04 [6]. Because of "the risk posed by chlorinated solvents in groundwater," it is a priority to "ensure no withdrawal or use of groundwater beneath the

site for anything other than environmental monitoring or testing until cleanup goals are met" [6]. The bio plugs contain a combination of three to four nonpathogenic species [6]. During installation the bio plugs are strategically inserted into the contaminated zone. The lower portion of the plugs consists of No. 10 well screen surrounded by coarse sand to prevent soil from entering and plugging the well screen slots. The inoculated media is located within the screened portion of the plug. During operation a small volume of water (1 to 5 gpd (gallons per day)/bio plug) containing nutrients (N, P, C amendment) is fed to the bio plugs, which in turn creates growth of microbes in the media and delivers the excess microbes that were sloughed off into the adjacent soil formation by a hydraulic pressure gradient. Air is fed into the bio plug, which keeps the bio plug aerobic and extends the oxygen rich water into the surrounding Upper Shallow Zone. In summary, the bio plug is a mini in situ biological reactor growing and delivering high concentrations of contaminant specific microbes into the contaminated zone. The microbes feed on the carbon in the CoC breaking apart the chemical structure resulting in the removal of the contaminant. The radius of impact for each bio plug is site-specific and dependent upon factors such as host matrix, hydraulic conductivity, percent water saturation, air and water flow rates [6]. The upper shallow zone where the contaminants are located has a high hydraulic conductivity [6]. 437 bio plugs were installed up to a depth of 30 feet with each having a radius of influence of 5 to 7.5 feet (Figures 6 and 7) [6]. The bio plugs are divided into eight zones with a zone control house and flow control attached to each bio plug in the zone [6]. There is baseline and quarterly monitoring of the groundwater [6]. The twelve monitoring wells are in four clusters of three wells [6]. Each of the three

wells is at different depth intervals to allow for lower, middle, and upper sampling of the upper shallow zone groundwater layer [6].

Figure 6. Monitoring Wells and Bio Plugs at Longhorn

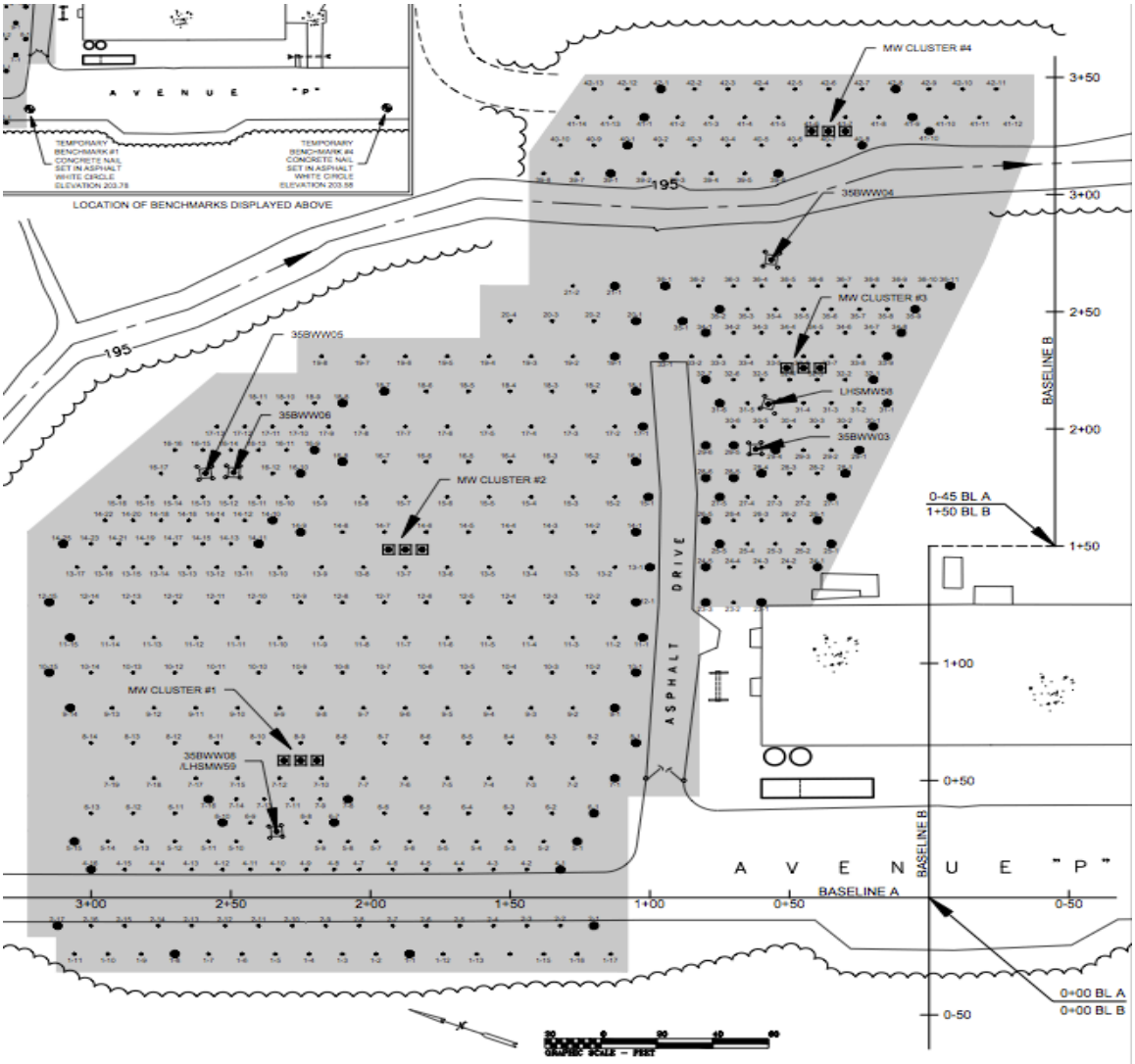


Figure 7. Longhorn Bioremediation Site Plan

2.3 Strategy for Microbial Community Structure

2.3.1 Community Structure Assessment

Biolog EcoPlates™ were used to conduct the community structure assessment. Previous journal articles on the Biolog EcoPlates™ have provided insight into the best methods for their use. The Biolog EcoPlates™ contain 31 different carbon substrates laced with tetrazolium violet dye. This "redox dye turns purple in the presence of electron transfer, indicating the substrate has been utilized by the inoculated microbes" (Sigler). Each substrate belongs to a carbohydrate, polymer, carboxylic acid, amino acid, amine or phenolic compound guild, shown in Table 2. Figure 8 shows the EcoPlate™ substrate layout.

Table 2. The EcoPlate™ Substrates and the Guilds to Which They Belong [23]

Well Number	Carbon Source	Guild
A1	Water (blank)	
B1	Pyruvic acid methyl ester	Carbohydrate
C1	Tween 40	Polymer
D1	Tween 80	Polymer
E1	Alpha-cyclodextrin	Polymer
F1	Glycogen	Polymer
G1	D-cellobiose	Carbohydrate
H1	Alpha-D-lactose	Carbohydrate
A2	Beta-methyl-D-glucoside	Carbohydrate
B2	D-xylose	Carbohydrate
C2	i-erythritol	Carbohydrate
D2	D-mannitol	Carbohydrate
E2	N-acetyl-D-glucosamine	Carbohydrate
F2	D-glucosaminic acid	Carboxylic acid
G2	Glucose-1-phosphate	Carbohydrate
H2	D,L-alpha-glycerol phosphate	Carbohydrate
A3	D-galactonic acid-gamma-lactone	Carboxylic acid
B3	D-galacturonic acid	Carboxylic acid
C3	2-Hydroxy benzoic acid	Phenolic Compound
D3	4-Hydroxy benzoic acid	Phenolic Compound
E3	Gamma-hydroxybutyric acid	Carboxylic acid
F3	Itaconic acid	Carboxylic acid
G3	Alpha-ketobutyric acid	Carboxylic acid
H3	D-malic acid	Carboxylic acid

(Table 2 continued)

Well Number	Carbon Source	Guild
A4	L-arginine	Amino Acid
B4	L-asparagine	Amino Acid
C4	L-phenylalanine	Amino Acid
D4	L-serine	Amino Acid
E4	L-threonine	Amino Acid
F4	Glycyl-L-glutamic acid	Amino Acid
G4	Phenylethylamine	Amine
H4	Putrescine	Amine

A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine	A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine	A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine
B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine	B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine	B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine
C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine	C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine	C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine
D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine	D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine	D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine
E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine	E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine	E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine
F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid	F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid	F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid
G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine	G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine	G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine
H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine	H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine	H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine

Figure 8. Carbon Sources on the EcoPlate™

Nine of the 31 substrates are compounds that are exuded from plant roots [14]. There are three control wells without a substrate on each plate. Therefore, any color development in these wells "presumably indicates utilization of carbon sources inherent in the inoculated water" [15]. There are three replicates of the substrates "within each EcoPlate™ to help account for variability in inoculum densities derived from environmental samples" [16]. The plates should be incubated "at temperatures ranging from 26 to 37˚C" [16]. To reduce desiccation some options include placing plates in polyethylene bags [16] or "plastic container[s] containing moist paper towels" [15]. From the multiple EcoPlate™

assays done for this research, the plates have lasted a little over 100 hours before desiccation began. According to Garland, "reduction of the tetrazolium dye does not occur until the cell numbers in the well reach about 10^5 cells/mL" [17]. The rate of utilization of different substrates also varies with different groups of microorganisms. This means there can be "high variability in the rate of [color] development and its intensity" [14]. The rate of color development depends on the number of cells and their metabolic activity [14].

One shortcoming of the EcoPlates™ is that the positive responses in the wells "indicate the ability of a community to adapt metabolism to an artificial (in vitro, not in situ) environment" [14]. Therefore, this "method indicates potential, but not actual, catabolic activity of a community" [14]. It is hard to identify which microorganisms contribute to substrate utilization on the EcoPlates™ "because of the huge microbial species diversity, different nutrient request and complicated between-species interactions" [14]. Stefanowicz thinks it is "mainly fast-growing bacteria, which are adapted to high substrate concentrations, especially if low density solution is inoculated, for example 10^4 CFU/ml" [14]. Therefore, "slow-growing bacteria, incapable of growing in such conditions, are not usually included in the analysis" [14].

An important variable to consider is that the "assortment of substrates does not necessarily reflect substrates which are available to bacteria in the soil environment, so one can suspect that some microbial species are incapable of growing on plates because of lack of proper substrates" [14]. It is also important to remember, "not all bacteria are able to reduce tetrazolium dye" [14]. Therefore, the "physiological profile obtained on a plate is a result of activity of only a part of microbial community and therefore one

should be careful when drawing conclusions about the structure or function of a whole community" [14]. Again, "the Biolog method investigates functional diversity or metabolic potential of that part of a community which is capable of being metabolically active and growing in plate conditions" [14]. This method should be used in comparative research. Ideally, these plates should be used to compare the "functional diversity of microbial communities from contaminated and non-contaminated soils" [14].

Various components describing community structure can be calculated from the absorbance values on the EcoPlates™. Average well color development (AWCD) "is a function of inoculum density and estimates the overall rate of color development" [18]. AWCD can be calculated using the following equation:

$$AWCD = \sum \frac{(C - R)}{n}$$

"where *C* is color production within each well (optical density measurement), *R* is the absorbance value of the plate's control well, and *n* is the number of substrates" [19]. Any negative values are set to zero [20]. A threshold optical density for a positive test must be established above which the purple coloration indicates a usage of the carbon substrate by the microbial community. This threshold is commonly set at 0.25 to eliminated weak false positive responses [18].

Some have gone an extra step and normalized the data by dividing each well optical density by the average well color development to diminish biases by different inoculum densities, especially if they were not normalized prior to inoculation [21]. Garland argues, "the effectiveness of dividing by AWCD is limited to samples with similar numbers of positive wells, since zero values cannot be effectively normalized" [18]. Normalization "is not necessary when general metabolic response of

microorganisms, for example, after exposure to contaminants is studied, or when communities from different types of soil are compared" [14]. Normalization becomes necessary when the goal is to understand the dynamics of a community composition [14]. Counting colony-forming units (CFU) on agar plates is one way to estimate the number of microorganisms in a sample [14]. One can also monitor color development over time and select plate readings with similar AWCD values.

The Shannon-Weaver Index is "a means of evaluating microbial community functional diversity" [21]. It is "a measure of the number of substrates utilized [indicated by a positive test] (substrate richness) and diversity of the extent of utilization of particular substrates (substrate evenness)" [14]. It can be calculated using the following equation:

$$H = -\sum_{i=1}^{N} p_i \, (\ln p_i)$$

"where p_i is the ratio of the corrected absorbance value of each well to the sum of absorbance value of all wells" [21] and "N is the number of substrates on a plate" [14].

The percent functional diversity is the percentage of the total number of substrates that are being used (indicated by a positive test). It can be calculated using the following equation:

$$\%\ Functional\ Diversity = \frac{number\ of\ positive\ carbon\ source\ wells}{n} * 100$$

The Shannon-Weaver index and percent functional diversity seem to be similar ways to calculate diversity. A high Shannon-Weaver index indicates a rich and evenly distributed community. A high percent functional diversity indicates a diverse community.

Percent variation of results within the sample is the variation between replicates on the EcoPlate™. It can be calculated using the following equation:

$$\%\ Variation\ of\ Results\ within\ Sample = \frac{i}{31} * 100$$

"where *i* is the number of carbon sources in which the three replicates were not all positive or all negative" [22].

The percent carbon substrate utilization was calculated by using the following equation:

$$S_x = \frac{10}{n} * \sum_{i=1}^{n} OD\ (substrate\ i)$$

where *i* is the substrate guild (i.e. substrate classification) and *n* is the number of substrates of each guild [20]. These values can then be converted to a percentage by dividing each guild value by the sum of all the guild values and multiplying by a hundred.

2.3.2 MicroPlate™ Approaches to Taxonomy

The MicroPlate™ assay identifies microorganisms from their phenotypic pattern. There are 94 phenotypic tests, as seen in Figure 9: 71 carbon source utilization assays and 23 chemical sensitivity assays. These assays work in the same way as the EcoPlates™.

A1 Negative Control	A2 Dextrin	A3 D-Maltose	A4 D-Trehalose	A5 D-Cellobiose	A6 Gentiobiose	A7 Sucrose	A8 D-Turanose	A9 Stachyose	A10 Positive Control	A11 pH 6	A12 pH 5
B1 D-Raffinose	B2 α-D-Lactose	B3 D-Melibiose	B4 β-Methyl-D-Glucoside	B5 D-Salicin	B6 N-Acetyl-D-Glucosamine	B7 N-Acetyl-β-D-Mannosamine	B8 N-Acetyl-D-Galactosamine	B9 N-Acetyl Neuraminic Acid	B10 1% NaCl	B11 4% NaCl	B12 8% NaCl
C1 α-D-Glucose	C2 D-Mannose	C3 D-Fructose	C4 D-Galactose	C5 3-Methyl Glucose	C6 D-Fucose	C7 L-Fucose	C8 L-Rhamnose	C9 Inosine	C10 1% Sodium Lactate	C11 Fusidic Acid	C12 D-Serine
D1 D-Sorbitol	D2 D-Mannitol	D3 D-Arabitol	D4 myo-Inositol	D5 Glycerol	D6 D-Glucose-6-PO4	D7 D-Fructose-6-PO4	D8 D-Aspartic Acid	D9 D-Serine	D10 Troleandomycin	D11 Rifamycin SV	D12 Minocycline
E1 Gelatin	E2 Glycyl-L-Proline	E3 L-Alanine	E4 L-Arginine	E5 L-Aspartic Acid	E6 L-Glutamic Acid	E7 L-Histidine	E8 L-Pyroglutamic Acid	E9 L-Serine	E10 Lincomycin	E11 Guanidine HCl	E12 Niaproof 4
F1 Pectin	F2 D-Galacturonic Acid	F3 L-Galactonic Acid Lactone	F4 D-Gluconic Acid	F5 D-Glucuronic Acid	F6 Glucuronamide	F7 Mucic Acid	F8 Quinic Acid	F9 D-Saccharic Acid	F10 Vancomycin	F11 Tetrazolium Violet	F12 Tetrazolium Blue
G1 p-Hydroxy-Phenylacetic Acid	G2 Methyl Pyruvate	G3 D-Lactic Acid Methyl Ester	G4 L-Lactic Acid	G5 Citric Acid	G6 α-Keto-Glutaric Acid	G7 D-Malic Acid	G8 L-Malic Acid	G9 Bromo-Succinic Acid	G10 Nalidixic Acid	G11 Lithium Chloride	G12 Potassium Tellurite
H1 Tween 40	H2 γ-Amino-Butyric Acid	H3 α-Hydroxy-Butyric Acid	H4 β-Hydroxy-D,L-Butyric Acid	H5 α-Keto-Butyric Acid	H6 Acetoacetic Acid	H7 Propionic Acid	H8 Acetic Acid	H9 Formic Acid	H10 Aztreonam	H11 Sodium Butyrate	H12 Sodium Bromate

Figure 9. Carbon Sources on the GEN III MicroPlate™

3 MATERIALS AND METHODS

3.1 Groundwater Sample Collection

These U.S. Department of Defense sites followed protocols outlined by the U.S. EPA for chemical analysis of water [24, 25]. Additionally, the Sunnyvale site follows sampling protocol specific to the Regional Water Quality Control Board as outlined by McLaren and Hart (1991) [26]. At the Longhorn site, there are additional protocols outlined by the U.S. Army Corps of Engineers for sampling [27]. A more detailed discussion of QA/QC was part of the federal and state permitting process.

3.2 Population Counts

Standard methods were used for culturing organisms for the purpose of counting the colony forming units. Minimal salts agar (MSA) was made by measuring out 1.5g KH_2PO_4, 1g yeast extract, 1g sodium extract, and 30g agar and adding it to 2L H_2O. It was constantly mixed using a magnetic stirrer and heated until completely dissolved. Agar was then autoclaved. Dilutions of samples with buffered water were made if necessary based on previous CFU counts. 1 mL aliquots of each sample were added to respective labeled petri dishes in triplicates (Figure 10). For Longhorn samples, 50 μL TCE was added to 500 mL cooled MSA as the sole carbon source. For Sunnyvale samples, 250 μL DCE was used instead. Agar was poured into the plates and swirled to mix with sample. Above steps were repeated using 23g nutrient agar suspended in 1L of purified water without added chlorinated aliphatics to find total heterotroph growth. Plates were regularly checked for growth and colonies counted under a microscope when growth appeared. The reported population counts are the average of the three counts (Figure 11).

Figure 10. Petri Dishes with Groundwater Samples Before Pouring Agar

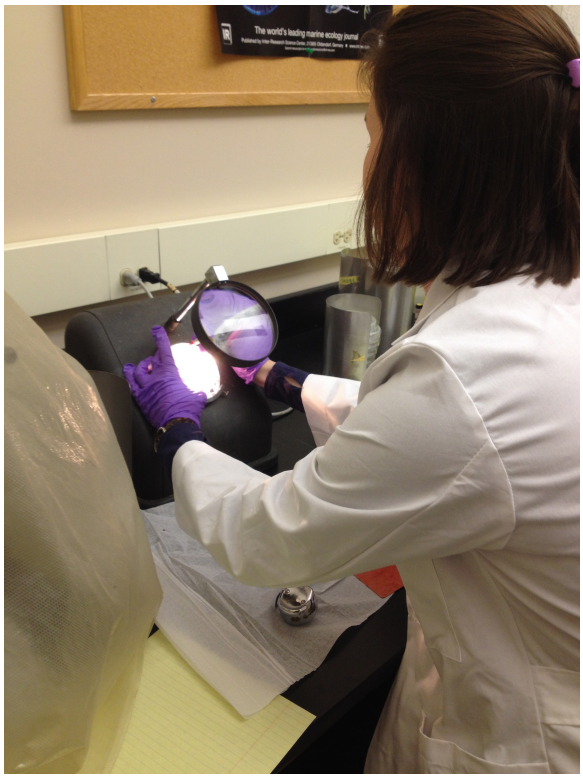


Figure 11. Counting Colonies

3.3 Microbial Identification

The Biolog MicroPlate™ protocol calls for culturing organisms on Biolog recommended agar media, preparing inoculum, inoculating the MicroPlates™, incubating the MicroPlates™, and reading and interpreting results. First, 57g of Biolog Dehydrated Growth Agar (BUG Agar) was mixed into one liter of purified water. It was constantly mixed using a magnetic stirrer and heated until completely dissolved. Agar was then autoclaved. Plates were poured and streaked with sample using Inoculatorz™ (sterile disposable inoculator swabs). Plates were incubated and a pure culture isolated. Biolog GEN III MicroPlates™ and Inoculating Fluid (IF-A) were brought to room temperature. Turbidimeter was blanked with a wiped IF tube each time and set to 100% transmittance. A small (3 mm) isolated colony was picked up with the Inoculatorz™. The swab was placed in the IF tube, rubbed against the bottom, and mixed. The wiped IF tube was placed back in the turbidimeter to confirm the transmittance was 90-98 %. The IF fluid was poured into a sterile disposable reservoir. 8 sterile pipette tips were attached to the Ovation™ 8 channel electronic repeating pipettor and filled with the IF fluid. 100 µL of fluid were released into each MicroPlate™ well. The MicroPlates™ were incubated in the Biolog OmniLog® incubator/reader. Clicking the 'Create Batch' button started the OmniLog™ software, selecting normal read mode (22 hour incubation, read and ID), labeling the plate position, and clicking 'Load Batch.' Batches were unloaded after the read was completed. See Appendix A for complete Biolog GEN III MicroPlate™ Instructions for Use.

3.4 Microbial Community Structure

Samples were poured into the reservoirs, as demonstrated in Figure 12. After attaching pipette tips to the electronic repeating pipettor, tips were filled and 100 µL of sample were released into each well of the Biolog EcoPlates™. EcoPlates™ were incubated in the Biolog OmniLog at 33˚ C, as seen in Figure 13. When it was time to read the plates, the Gen5 software was started and a protocol created for a 96 well plate with no lid and absorbance recorded at 540 nm. After the EcoPlate™ was placed in the Biolog MicroStation™ reader, the 'Read New' button was clicked and the data was exported to an Excel spreadsheet. From the Excel spreadsheet, calculations were made. EcoPlates™ were read every 24 hours for 5 to 6 days. See Appendix B for complete Biolog EcoPlate™ information sheet.

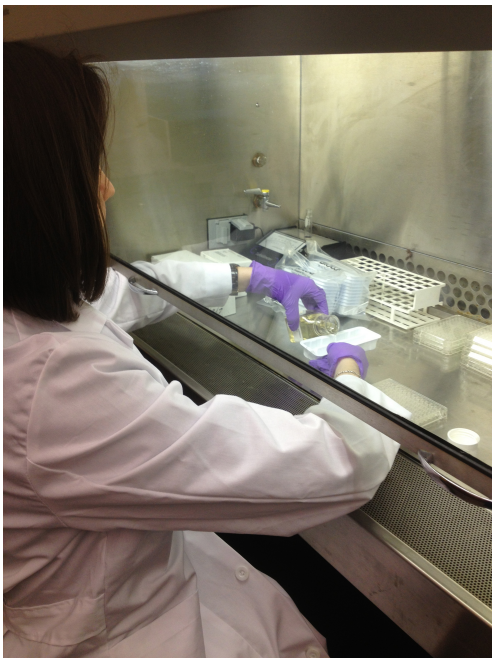
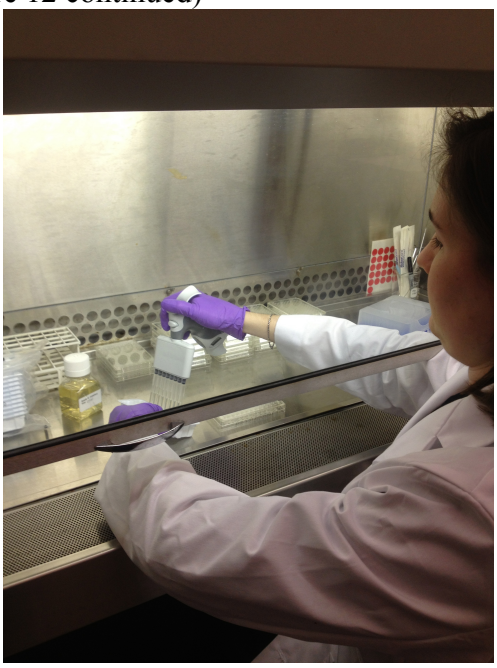
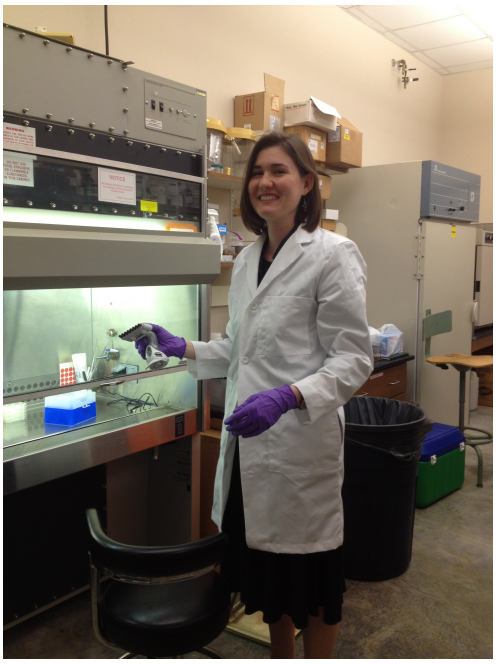
a)  b) 

Figure 12. Pipetting Groundwater Samples into EcoPlates™ (a-d)

(Figure 12 continued)

c)  d) 

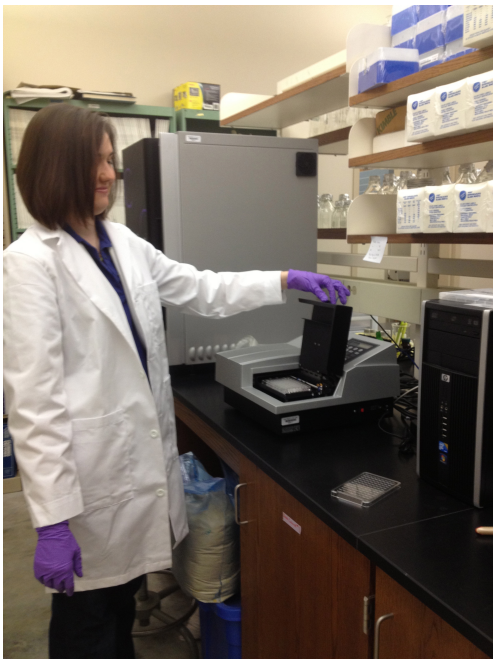
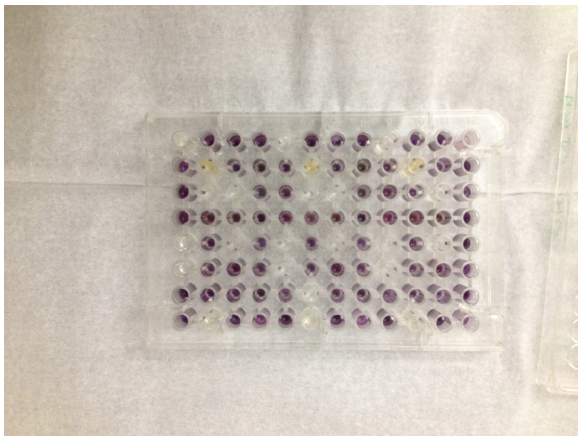
a)  b) 

Figure 13. a) EcoPlate™ Incubation, b) Reading EcoPlates™, and c) EcoPlate™ without Cover after Incubation

(Figure 13 continued)

c) 

3.5 Acute Toxicity Test

A MicroTox® machine was used to conduct acute toxicity tests on groundwater samples from each site, using the bioluminescent bacteria *Vibrio fischeri* to measure metabolic inhibition [28]. Samples 51, 68, and 70 from LMSSC and samples MW58, WW04, and WW12 from LHAAP were tested using the basic test protocol with four dilutions and an initial concentration of 45% [28]. A zinc sulfate standard was used. Table shows results at time zero before standard was added and results 15 minutes after standard was added. The light intensity, I, is measured in absolute light units [28]. Due to the large decrease in light intensity, the zinc sulfate standard is toxic (Table 3).

Table 3. Zinc Sulfate Standard

Standard Concentration (mg/L)	I_0	I_{15}
0	93	75
5.63	102	7
11.25	90	3
22.5	79	2
45	103	2

4 RESULTS AND DISCUSSION

4.1 Assessment of Sunnyvale *in situ* Microbial Communities

4.1.1 TCE Concentrations in Site Groundwater

TCE concentrations have fluctuated over time. In the Intermediate Transmissive Zone, the concentrations are on the decline. The decrease is statistically significant overtime with a p-value of 0.00002. The 2B (p=0.40844) and 2nd (p=0.28794) Transmissive Zones have somewhat stable concentrations and do not have statistically significant change overtime. Table 4 and Figure 14 show the values for each zone from March 2009 through January 2014.

Table 4. Average TCE Concentrations in µg/L for Each Transmissive Zone: Sunnyvale

	2nd Zone	2B Zone	Intermediate Zone
March 2009	11392.86	11505.64	52844.40
May 2009	13497.14	13097.84	61645.79
June 2009	13622.86	13185.89	65054.57
July 2009	11244.29	12813.52	67749.08
August 2009	7650.00	13197.23	59529.31
September 2009	6821.43	15041.99	76930.17
October 2009	8981.43	11833.86	41546.21
November 2009	12945.71	14442.97	46297.35
December 2009	20458.75	10974.85	41959.50
January 2010	20627.50	12203.96	33071.17
April 2010	19918.75	9707.55	34610.34
July 2010	21806.25	9961.12	43710.31
October 2010	17995.00	11413.88	60368.60
January 2011	15377.50	9196.70	54142.79
April 2011	15031.25	9132.03	39358.75
July 2011	14051.25	7345.60	31253.66
October 2011	23948.38	9021.04	38470.51
January 2012	12980.38	13707.61	49529.50
April 2012	13560.75	12110.90	31359.46
July 2012	9922.38	15027.65	40152.38
October 2012	11912.25	12136.75	32399.75
January 2013	11769.00	10726.74	27027.63
April 2013	12008.13	11441.79	28777.13
July 2013	12171.88	11270.15	29773.75
October 2013	8022.50	13123.33	28024.25

(Table 4 continued)

	2nd Zone	2B Zone	Intermediate Zone
January 2014	9335.88	10790.97	23218.18

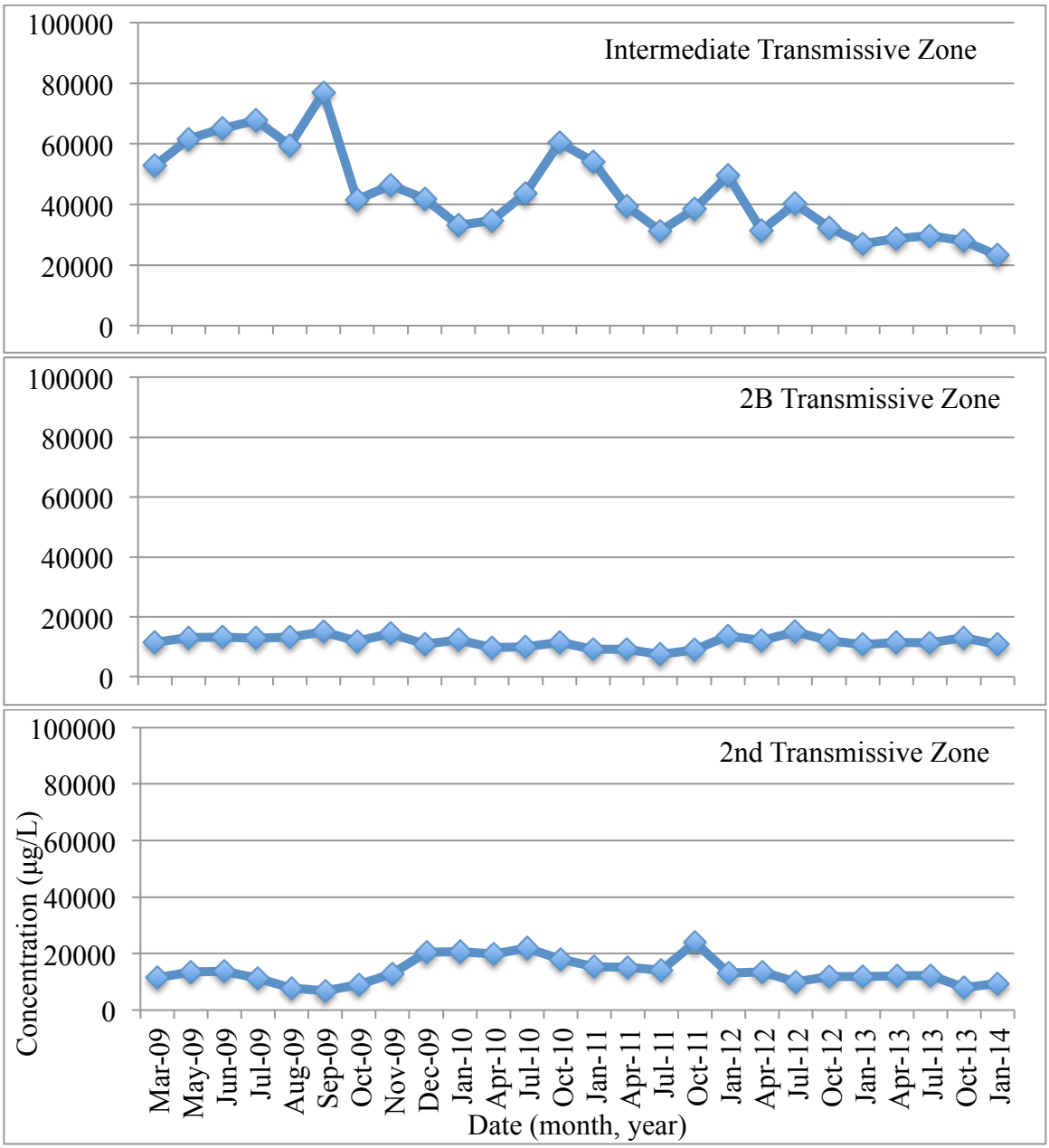


Figure 14. Temporal Change in Average TCE Concentrations for Intermediate, 2B, and 2nd Transmissive Zones: Sunnyvale

4.1.2 Chlorinated Aliphatic Hydrocarbon Degrader Plate Counts

Figure 15 shows the population counts of the chlorinated aliphatic degraders. In August 2012, only 14 samples were collected at the Sunnyvale site. Monitoring well 70

and 75 had values of zero. Monitoring well 71 had the highest colony count. In January 2013, 30 samples were collected. Monitoring wells 21, 59, and 66 had values of zero. Monitoring well 56 had the highest colony count. In July 2013, 30 samples were collected. Monitoring wells 65, 70 and 73 had values of zero. Monitoring well 56 again had the highest count. In January 2014, 30 samples were collected and all showed growth. Monitoring well 65 had the highest count. The colony counts have fluctuated over this time period. The average CFU/ml was 297 in August 2012, 237 in January 2013, 12 in July 2013, and 111 in January 2014. Note, the large gap in results of August 2012 samples 49-66 is due to a lack of sampling not from a result of 0 CFU/ml.

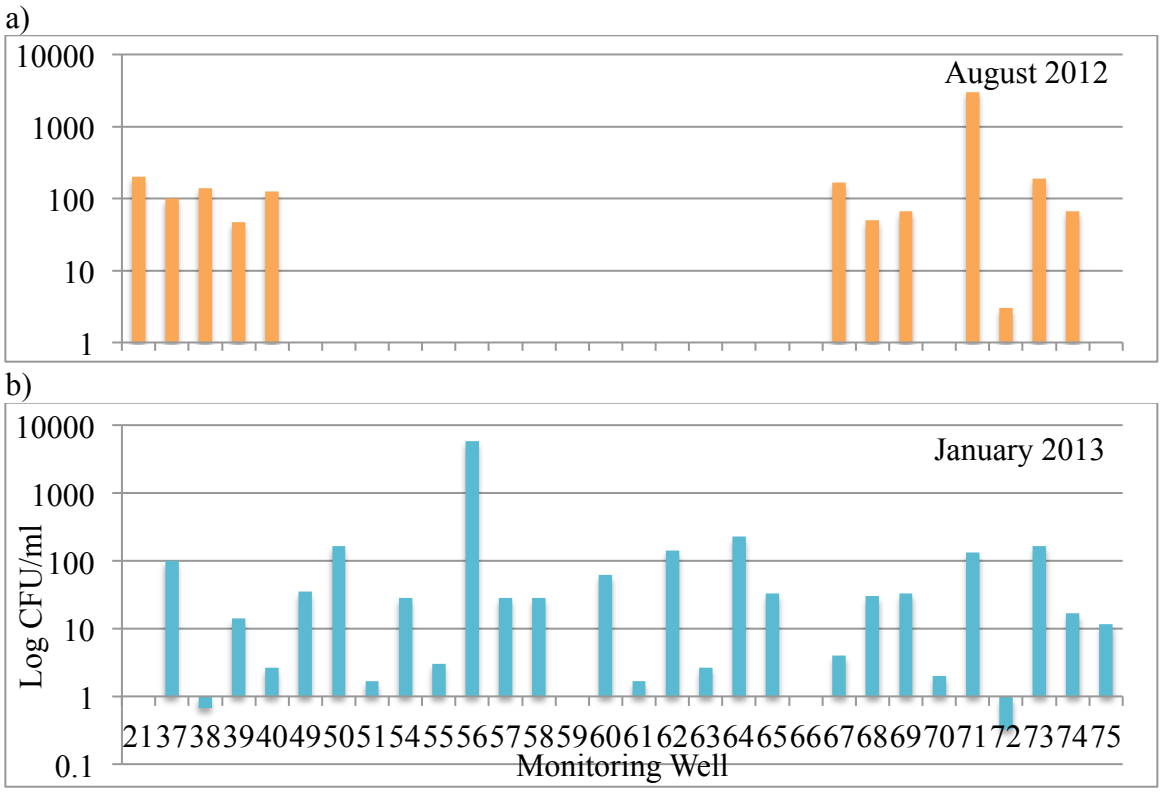
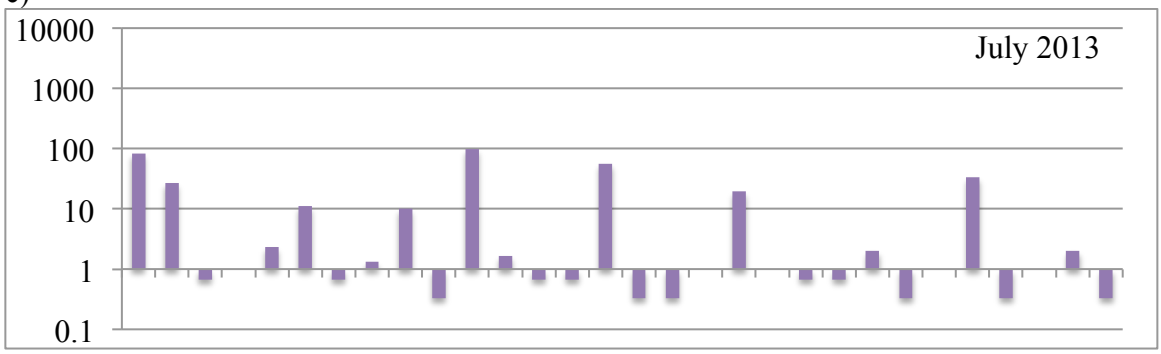


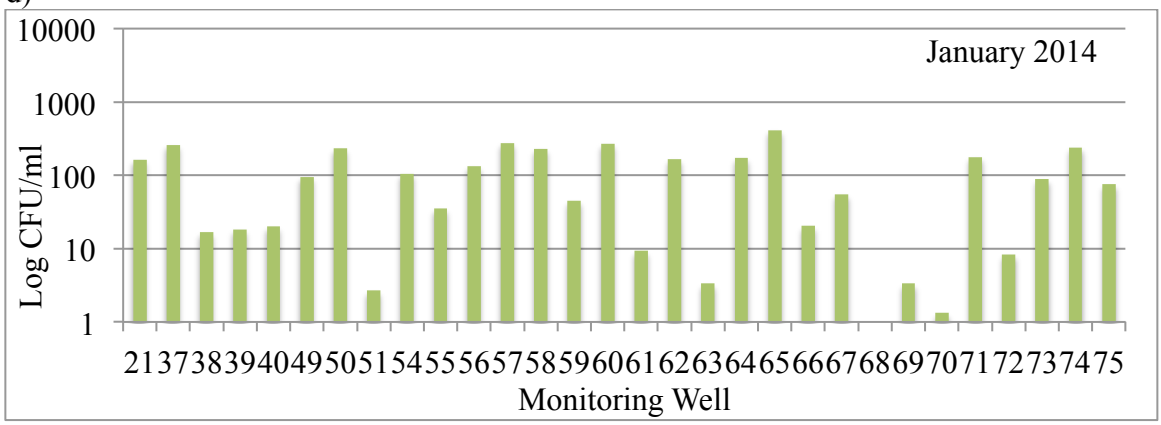
Figure 15. Logarithmic Transformation of the Calculated Final Colony Counts of Chlorinated Aliphatic Hydrocarbon Degraders from August 2012 to January 2014 (a-d): Sunnyvale

(Figure 15 continued)

c)



d)



4.1.3 Heterotroph Plate Counts

Heterotroph colony counts were collected for July 2013 and January 2014 as seen in Figure 16.

a)

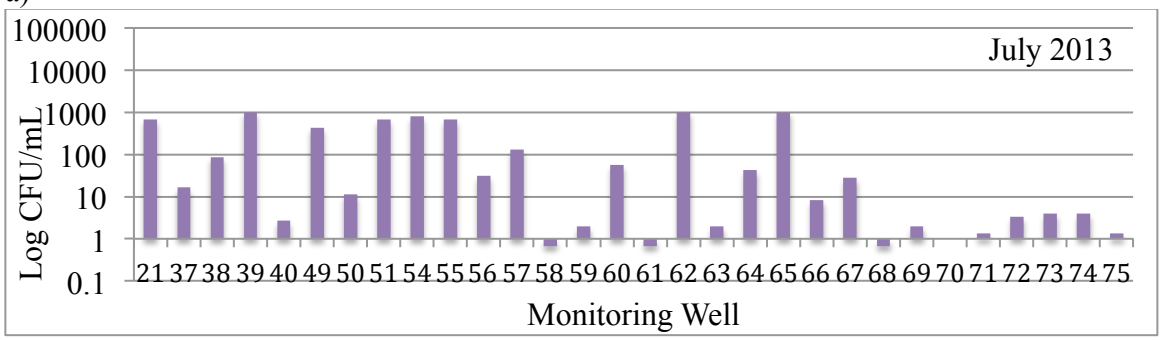
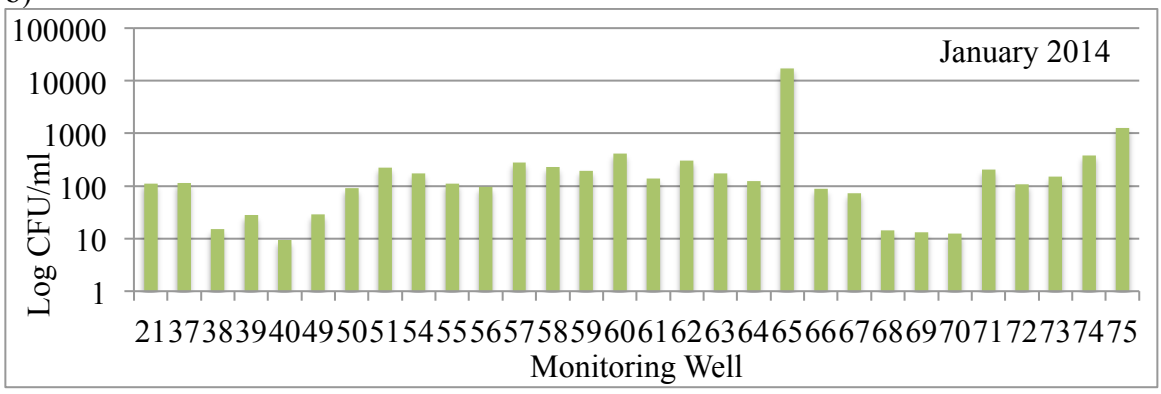


Figure 16. Logarithmic Transformation of the Calculated Final Colony Counts of Heterotrophs for a) July 2013 and b) January 2014: Sunnyvale

(Figure 16 continued)

b)



Monitoring wells 39, 62 and 65 tied for the highest counts in July 2013. Monitoring well 70 had a value of zero. Monitoring well 65 had the highest value in January 2014. The counts have increased in 73% of the wells from July 2013 to January 2014. The average CFU/ml was 222 in July 2013 and 748 in January 2014.

4.1.4 Average Well Color Development

The AWCD estimates the overall rate of color development. In July 2013, monitoring well 56 had the highest AWCD (Figure 17). Appropriately, it also had the highest number of CFUs/ml out of all the samples. In January 2014, monitoring well 56 again had the highest AWCD.

a)

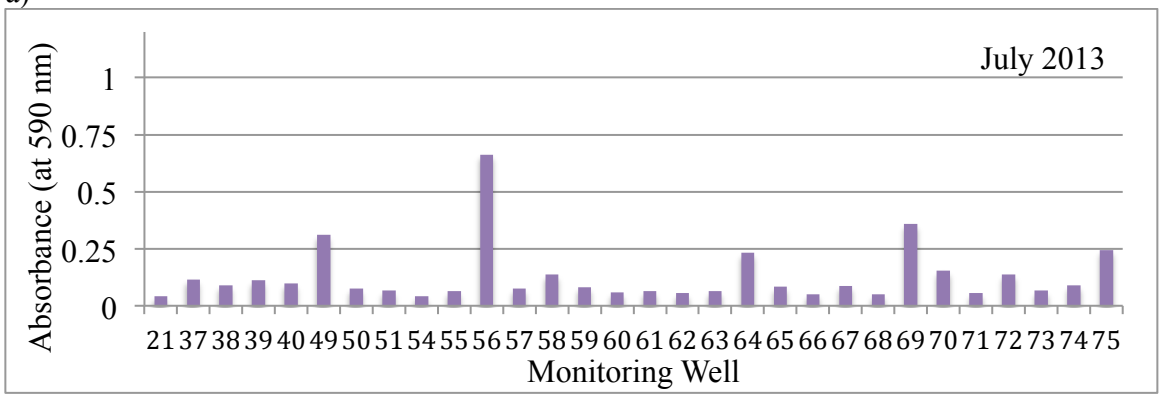
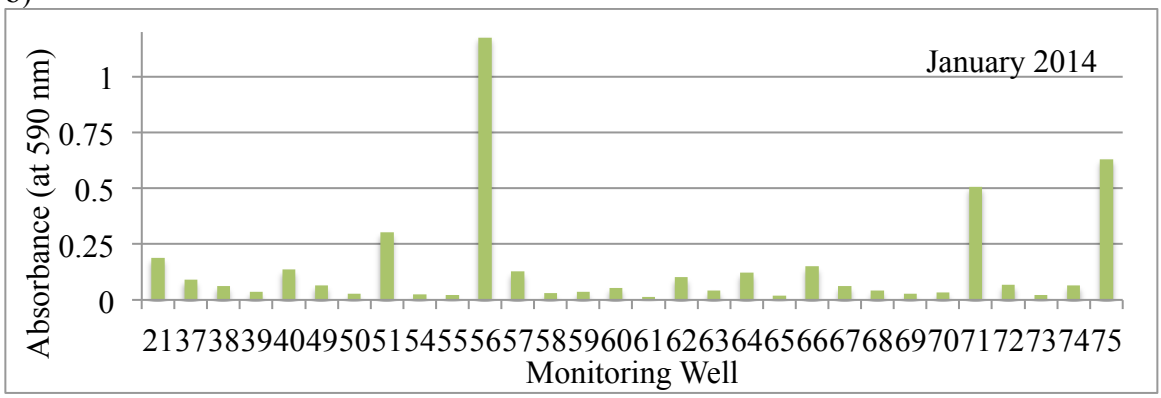


Figure 17. Average Well Color Development of a) July 2013 and b) January 2014 Samples: Sunnyvale

(Figure 17 continued)

b)



4.1.5 Functional Diversity

Figure 18 shows the percent functional diversity of each sample over the incubation time.

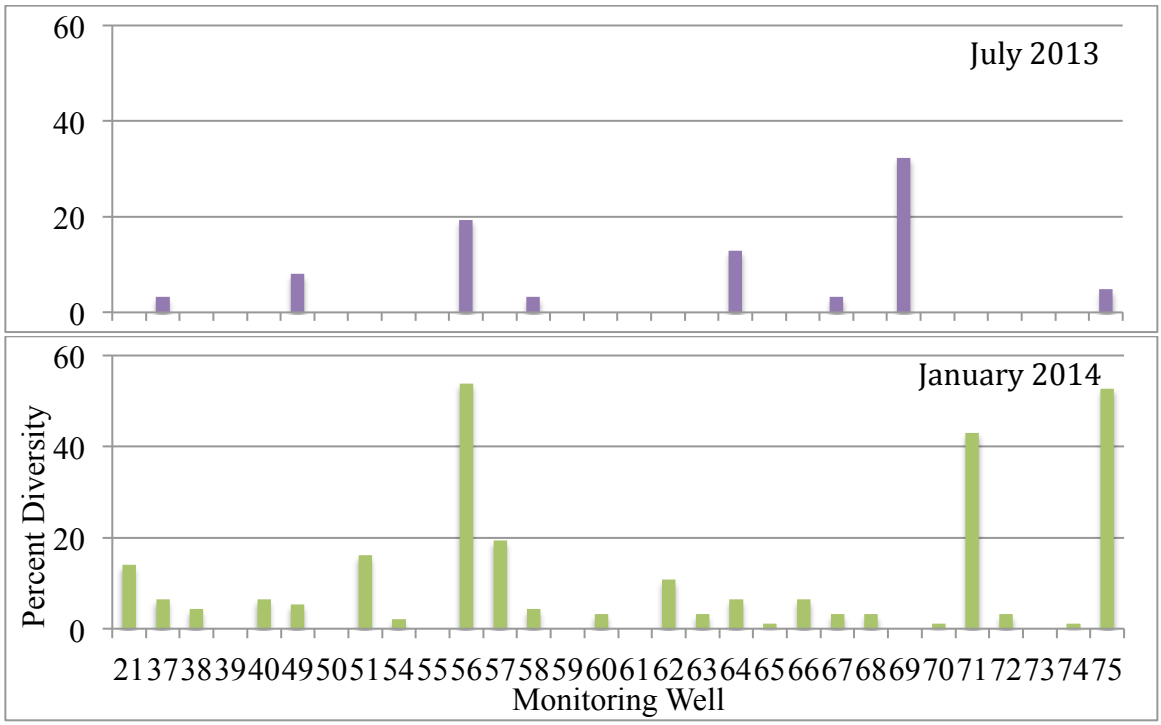


Figure 18. Percent Functional Diversity of July 2013 and January 2014 Samples: Sunnyvale

In July 2013, of the 30 monitoring wells sampled, only 8 showed presence of microorganisms on the EcoPlate™ assays. Naturally, all but eight of the wells have zero diversity and all have very low abundance (reflected in AWCD results). Monitoring well 69 had the highest percent functional diversity and Shannon-Weaver index (see below). A high diversity is reflected in the even distribution of the various carbon sources used as shown in Figure 21. In January 2014, the samples 56, 71, 75 also have the highest AWCD, functional diversity, and Shannon-Weaver index of the group, as seen in Figures 17, 18, and 19, respectively. The diversity is also seen in Figure 21 where they are the samples with the most even distribution of utilized substrates. When comparing the results between July 2013 and January 2014, the January 2014 microbial communities are more diverse and abundant.

4.1.6 Shannon-Weaver Index

Figure 19 shows the Shannon-Weaver diversity index measuring richness and evenness. In July 2013, only four samples showed microbial growth that was picked up on the EcoPlates™. There is an obvious increase in diversity in January 2014.

a)

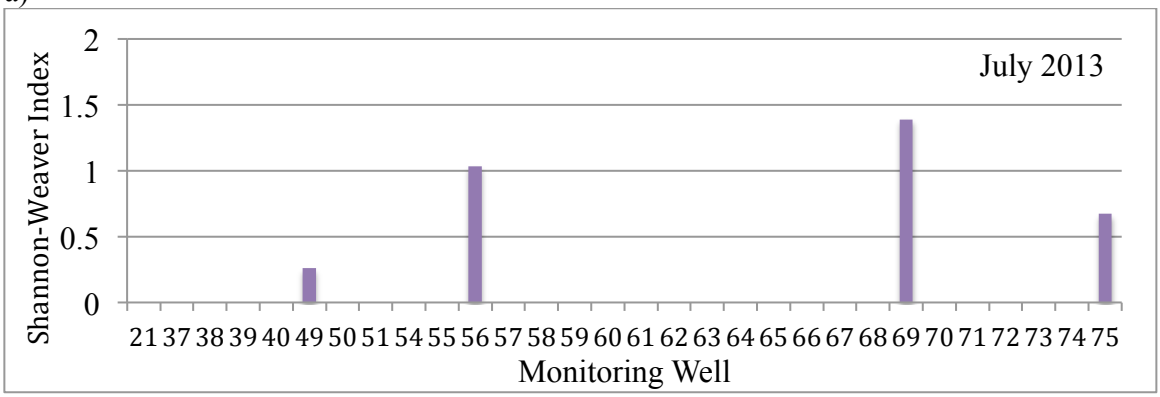
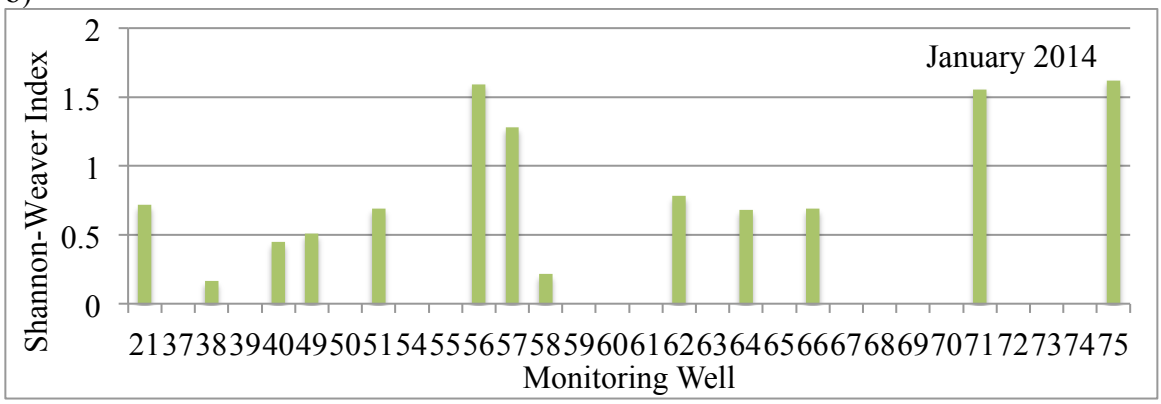


Figure 19. Shannon-Weaver Index for a) July 2013 and b) January 2014 Samples: Sunnyvale

(Figure 19 continued)

b)



4.1.7 Variation in EcoPlate™ Replicates

Figure 20 shows the percent variation among the three replicates on an EcoPlate™. Ideally, there should be no variation among replicates. There is a decrease in sample replicate variation over the sampling events, meaning the EcoPlate™ results are more precise.

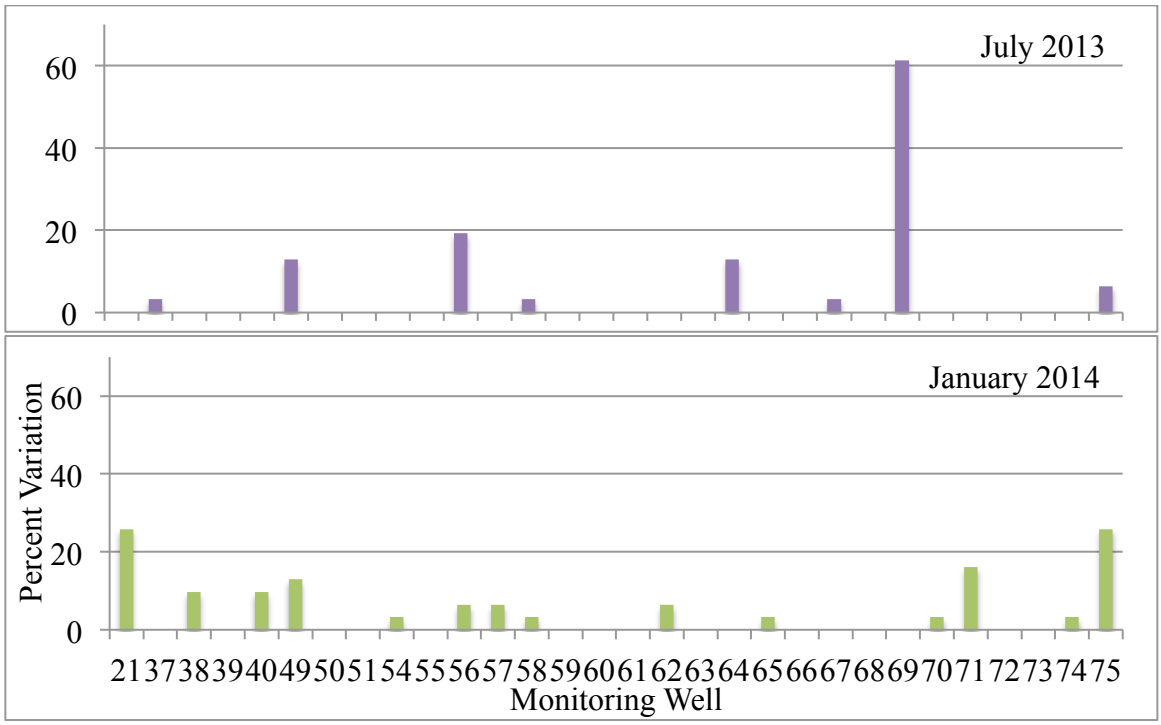


Figure 20. Percent Variation in Replicates within Each Sample for July 2013 and January 2014: Sunnyvale

4.1.8 Carbon Substrates Used

Figure 21 shows July 2013 and January 2014 substrate utilization.

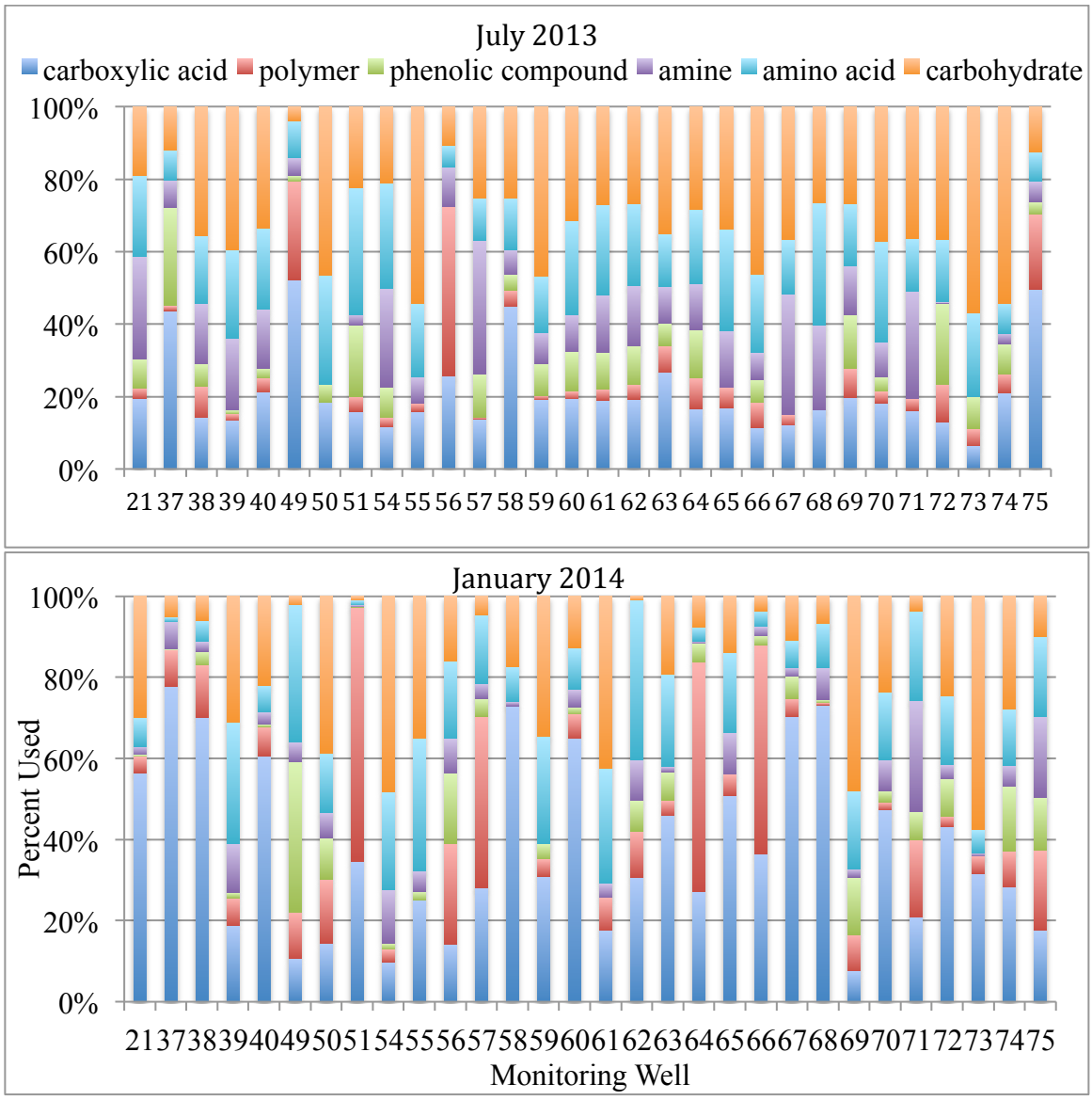


Figure 21. Percent of Carbon Substrates Used in July 2013 and January 2014: Sunnyvale

In July 2013, the substrate utilization appears to be more evenly distributed across the substrate options. In January 2014, the percentage of carbon substrates utilized greatly differs among the wells. Monitoring wells 56, 71 and 75 had the most even distribution of substrate usage for January 2014. The phenolic compound usage in July 2013 was non-existent for monitoring wells 56 and 71. All three monitoring wells increased their

phenolic compound usage in January 2014. Figure 22 shows the changes in substrate utilization from January 2013 to January 2014 for monitoring wells 60, 62, and 64.

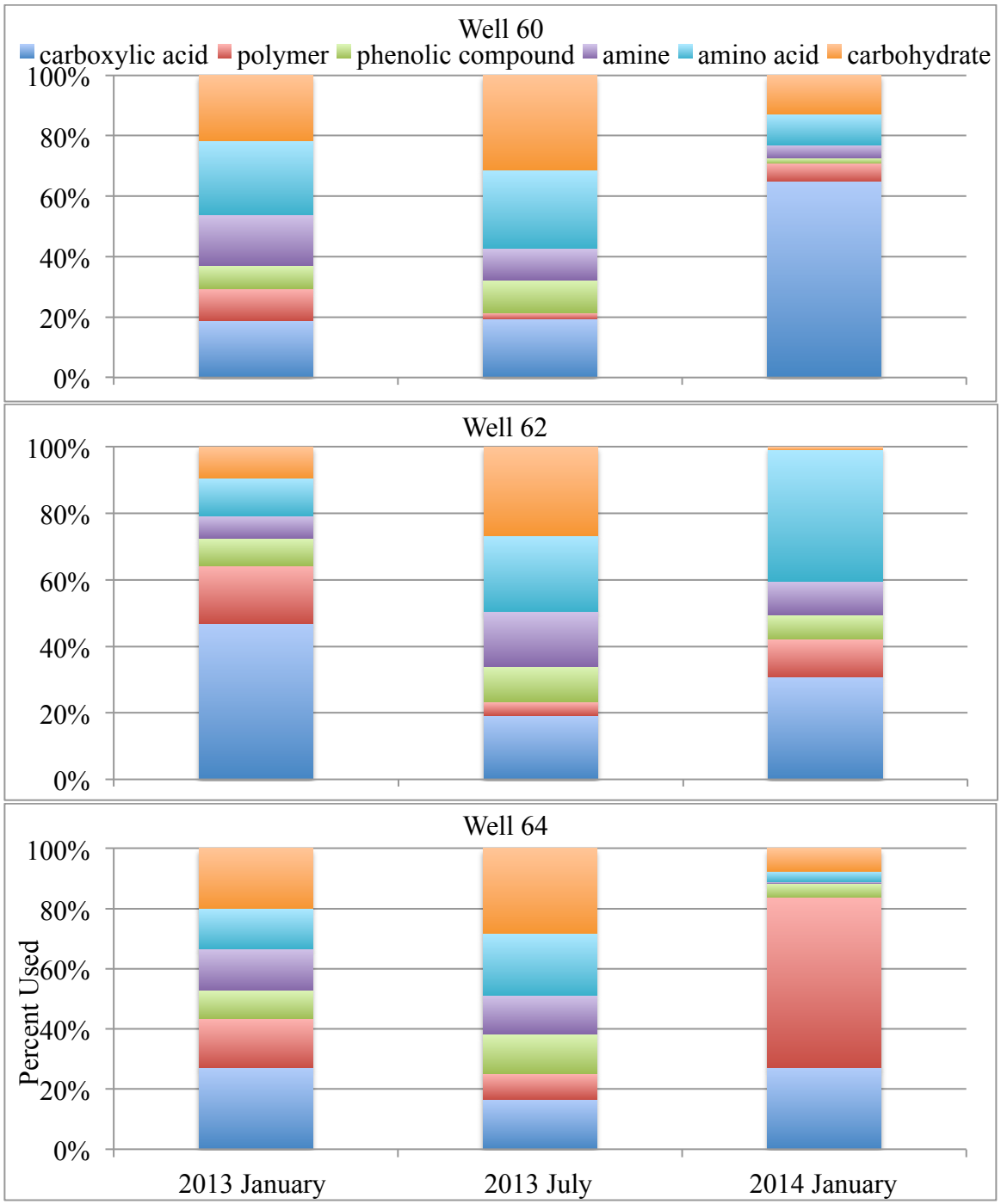


Figure 22. Temporal Change in Percent of Carbon Substrates Used for Monitoring Wells 60, 62, and 64 from January 2013 to January 2014: Sunnyvale

4.1.9 Species Identification

In January 2013, samples of bio plug pellets used at Sunnyvale were collected, suspended in water, and incubated to grow out the microorganisms for testing. The MicroPlate™ assay concluded that the pellets contain *Ochrobactrum intermedium* and *Ochrobactrum tritici* bacteria. Unfortunately, the pellet samples were taken off the top of the bio plug and not from the middle so the species present were not the ones that were initially there. The bio plugs were inoculated with *Acinetobacter haemolyticus*, *Pseudomonas aeruginosa*, and *Pseudomonas stutzeri*. In July 2013, the species found in monitoring well 38 was *Micrococcus luteus B*. Of the samples 39, 38, 57, and 75 used to find the bacterial species present, only 38 had growth. In January 2014, the MicroPlates™ identified *Cupriavidus necator* (renamed *Ralstonia eutropha*) as the species found in well 74. All these species are endogenous to soil, though they are not known to be chlorinated aliphatic degraders.

4.1.10 Acute Toxicity Test

MicroTox® acute toxicity tests were run on January 2014 samples 51, 68 and 70. Tables 5 through 7 show results at time zero before samples were added and 15 minutes after samples were added. The light intensity, I, is measured in absolute light units. Adding the samples barely had an effect on the light intensity. Therefore, the samples are not toxic.

Table 5. Toxicity of Monitoring Well 51: Sunnyvale Site

Sample Concentration (mg/L)	I_0	I_{15}
0	83	70
5.63	80	70
11.25	84	72
22.5	83	70
45	80	68

Table 6. Toxicity of Monitoring Well 68: Sunnyvale Site

Sample Concentration (mg/L)	I_0	I_{15}
0	92	88
5.63	94	88
11.25	91	87
22.5	87	83
45	81	78

Table 7. Toxicity of Monitoring Well 70: Sunnyvale Site

Sample Concentration (mg/L)	I_0	I_{15}
0	91	81
5.63	134	117
11.25	130	116
22.5	126	111
45	116	103

4.2 Assessment of Longhorn *in situ* Microbial Communities

4.2.1 TCE Concentrations in Site Groundwater

As seen in Table 8, the TCE concentrations show a declining trend, but not a statistically significant one (p=0.08598). The largest decline in concentration since July 2012 was in WW08. Note, ND means non-detect and NS means not sampled.

Table 8. TCE Concentrations in µg/L: Longhorn

	July 2012	December 2012	March 2013	June 2013	September 2013	December 2013	March 2014
WW01	ND	NS	NS	NS	NS	NS	NS
WW02	NS	NS	NS	NS	NS	NS	NS
WW03	ND	ND	NS	ND	NS	ND	ND
WW04	8.09	NS	15.40	11.40	13.40	9.39	4.53
WW05	13.50	14.60	15.60	18.80	17.30	13.00	15.1
WW06	ND	ND	ND	NS	NS	ND	ND
WW07	ND	NS	NS	NS	ND	NS	NS
WW08	65.70	62.50	77.10	67.50	49.60	39.70	31
WW09	55.60	NS	45.20	43.90	53.60	54.30	73.3
WW10						54.4	37.2
WW11	ND	NS	NS	NS	ND	NS	NS
WW12						2.40	NS
WW14	80.60	80.50	80.30	90.90	89.70	72.60	NS
WW17						4.53	0.349

(Table 8 continued)

	July 2012	December 2012	March 2013	June 2013	September 2013	December 2013	March 2014
WW20						7.33	NS
MW58	5.17	6.76	3.39	5.02	4.99	1.57	0.312
MW1-1	16.80	17.60	24.80	22.1	4.83	5.09	2.82
MW1-2	8.66	8.57	8.96	9.13	0.817	ND	ND
MW1-3	2.80	NS	NS	NS	NS	NS	0.426
MW2-1	4.59	7.25	4.78	3.35	2.15	1.82	1.37
MW2-2	0.27	0.272	0.312	0.320	ND	ND	ND
MW2-3	1.44	1.24	0.915	0.840	0.289	ND	0.259
MW3-1	2.42	2.43	2.57	2.02	2.05	1.84	1.86
MW3-2	3.07	1.90	2.14	2.71	3.02	3.42	2.49
MW3-3	5.99	5.79	5.49	7.64	5.50	5.28	2.68
MW4-1	3.63	4.16	4.27	5.32	5.48	4.38	5.13
MW4-2	4.21	4.19	3.77	4.53	6.14	7.21	6.28
MW4-3	13.50	13.5	12.40	4.97	5.53	NS	3.38

4.2.2 Chlorinated Aliphatic Hydrocarbon Degrader Plate Counts

Figure 23 shows the calculated counts of colony-forming units that degrade chlorinated aliphatic hydrocarbons. In July 2012, 23 samples arrived and all showed growth. In September 2012, of the 21 samples that arrived, only one, MW 3-1, did not have any growth. In December 2012, of the 15 samples that arrived, 6 had no growth. The numbers decreased since September 2012. In March 2013, 18 samples arrived and all showed growth. The maximum counts decreased since December 2012, but more samples had growth. In June 2013, 18 samples arrived and only one, MW 3-2, did not have any growth. The numbers decreased since March 2013. In September 2013, 19 samples arrived and all had growth. In December 2013, 22 samples arrived and only one, WW03, did not have any growth. The average CFU/ml was 3145 in July 2012, 84979 in September 2012, 465 in December 2012, 42 in March 2013, 13 in June 2013, 73 in September 2013, and 234 in December 2013.

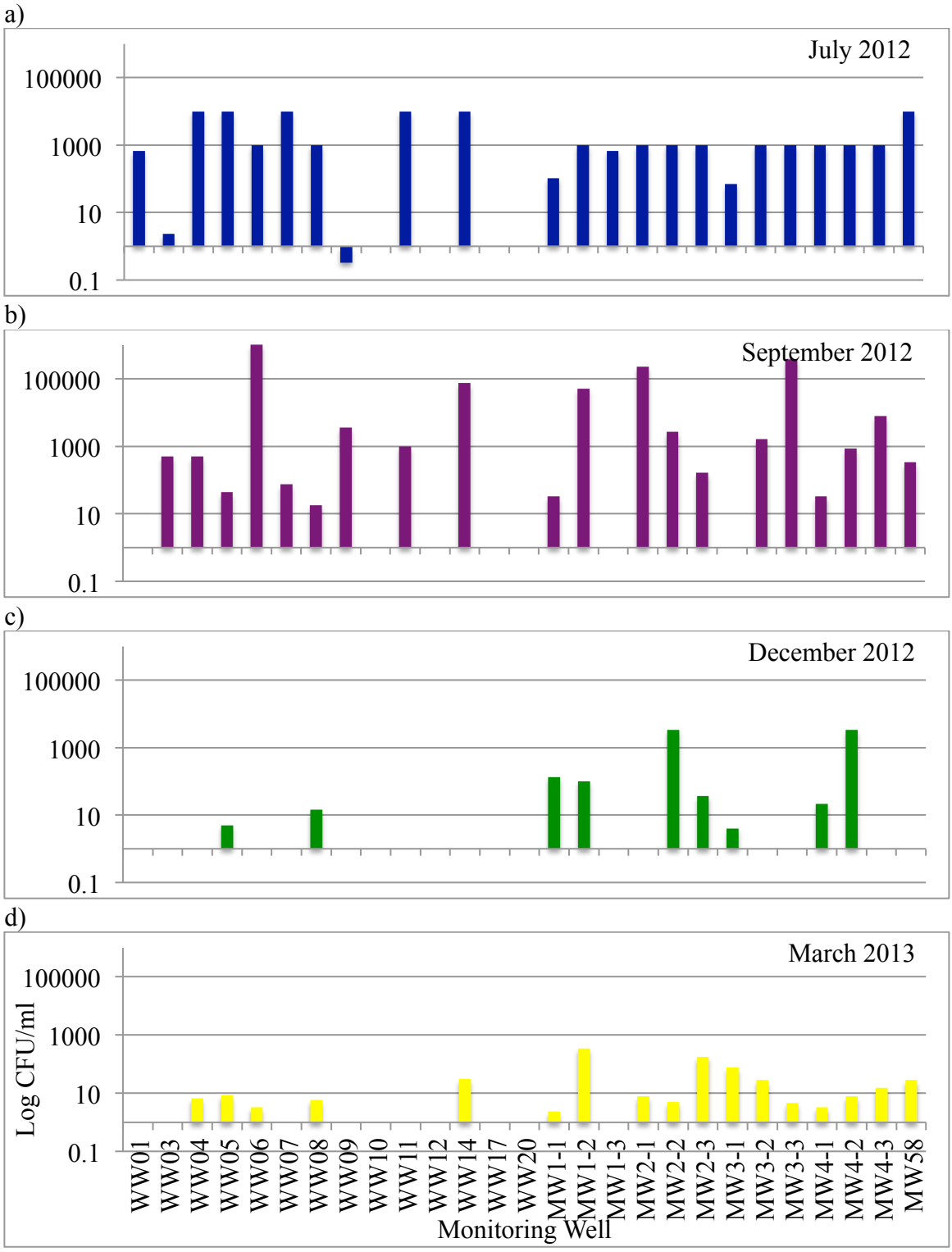
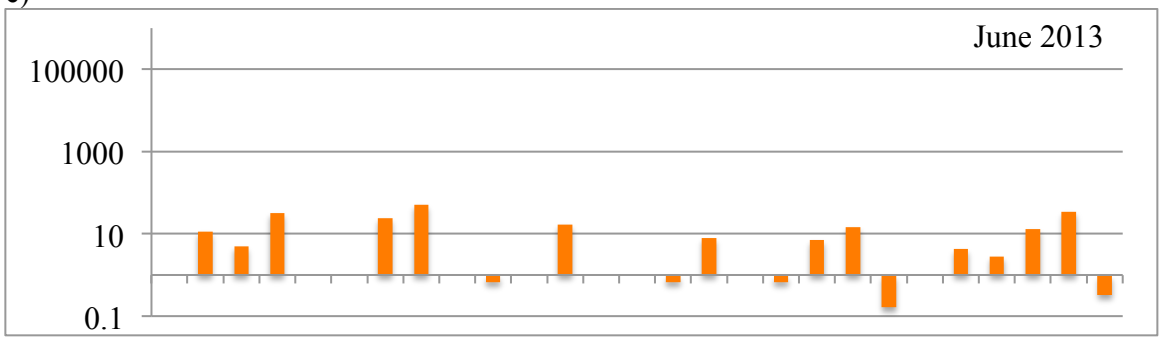


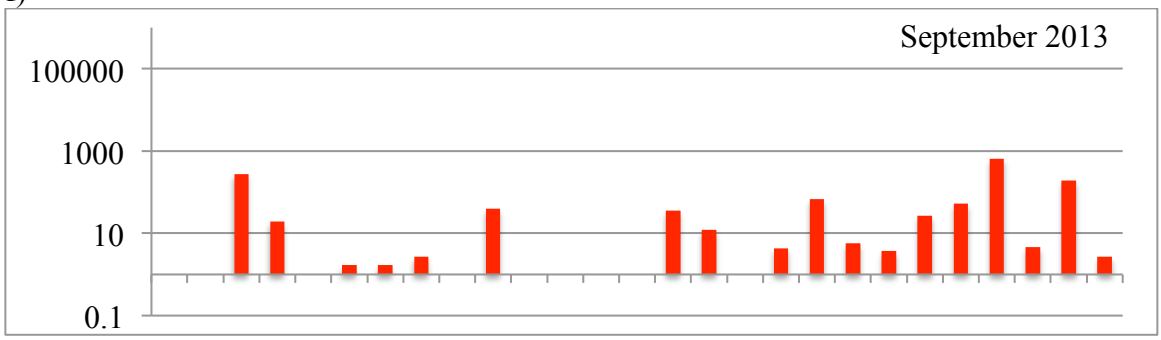
Figure 23. Logarithmic Transformation of the Calculated Final Colony Counts of Chlorinated Aliphatic Hydrocarbon Degraders from July 2012 to December 2013 (a-g): Longhorn

(Figure 23 continued)

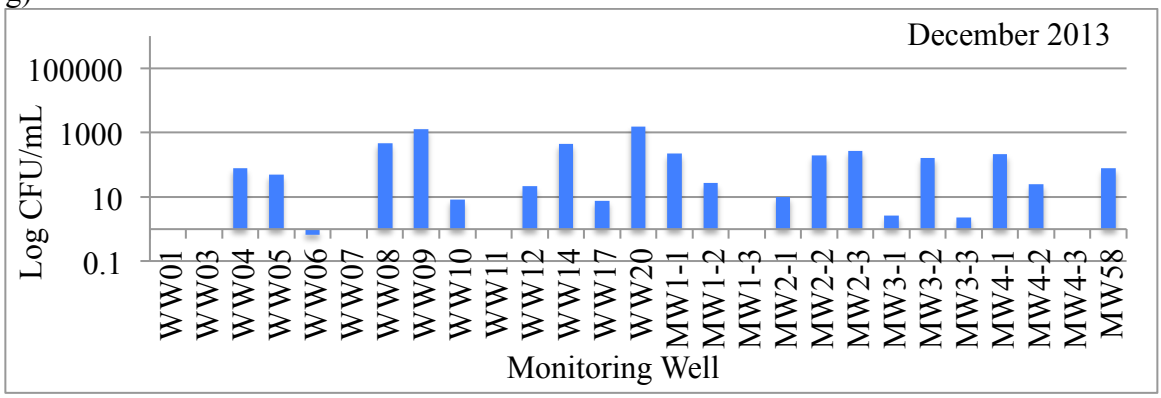
e)



f)



g)



4.2.3 Heterotroph Plate Counts

Figure 24 shows the calculated counts of heterotroph colony-forming units. June 2013 had the lowest counts, but they have since increased. The average CFU/ml was 3502 in July 2012, 166595 in September 2012, 39 in June 2013, 3231 in September 2013, and 5226 in December 2013. Note, all samples had growth, so any blanks indicate that sample was not collected.

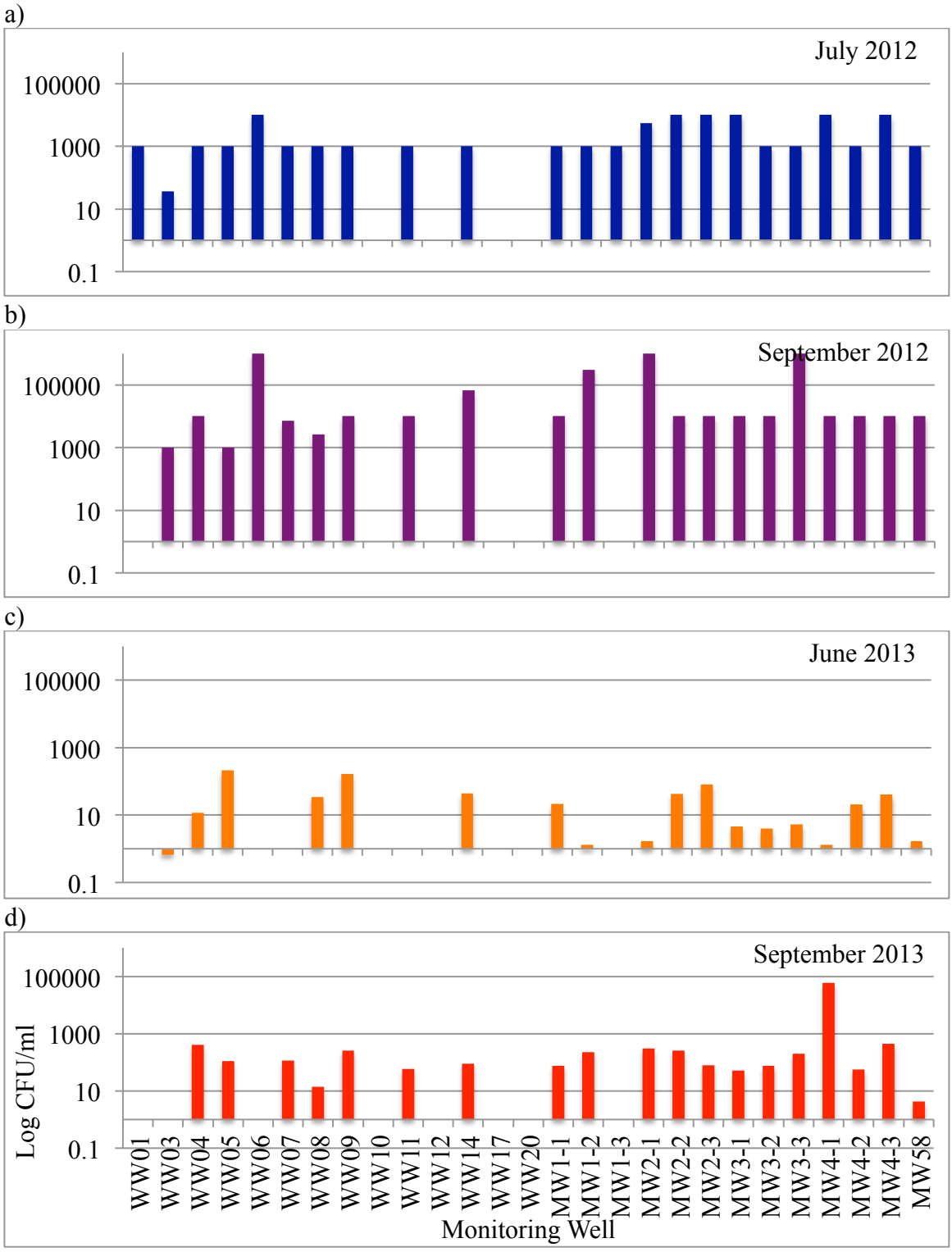
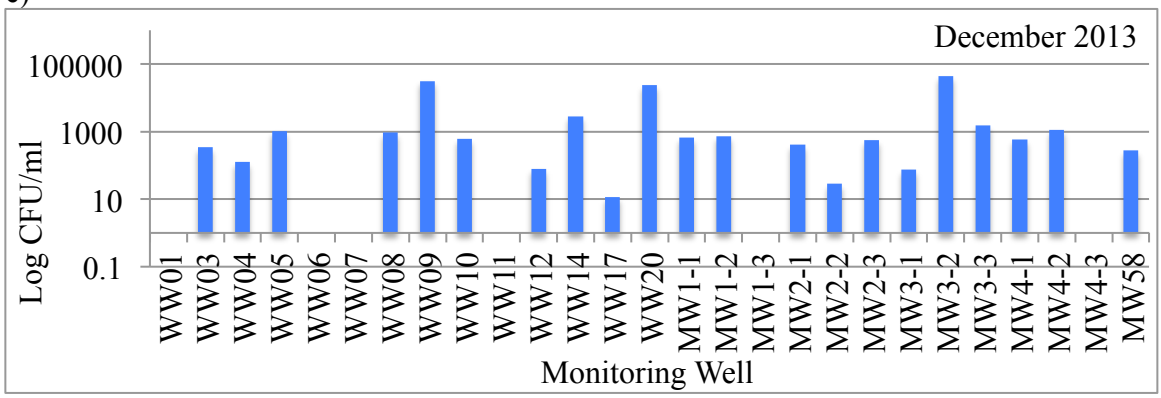


Figure 24. Logarithmic Transformation of the Calculated Final Colony Counts of Heterotrophs from June 2013 to December 2013 (a-e): Longhorn

(Figure 24 continued)

e)



4.2.4 Average Well Color Development

The absorbance value increases as the wells on the EcoPlate™ change color in the presence of microbes. In September 2013, MW4-3, as seen in Figure 25, has the highest abundance due to the fact that it has the highest absorbance value. In December 2013, the AWCD is varied across the samples. Samples MW2-3, WW04, WW05, and WW20 have the highest values. Note, for all of the following microbial community analysis, there is variation in which samples were collected and analyzed.

a)

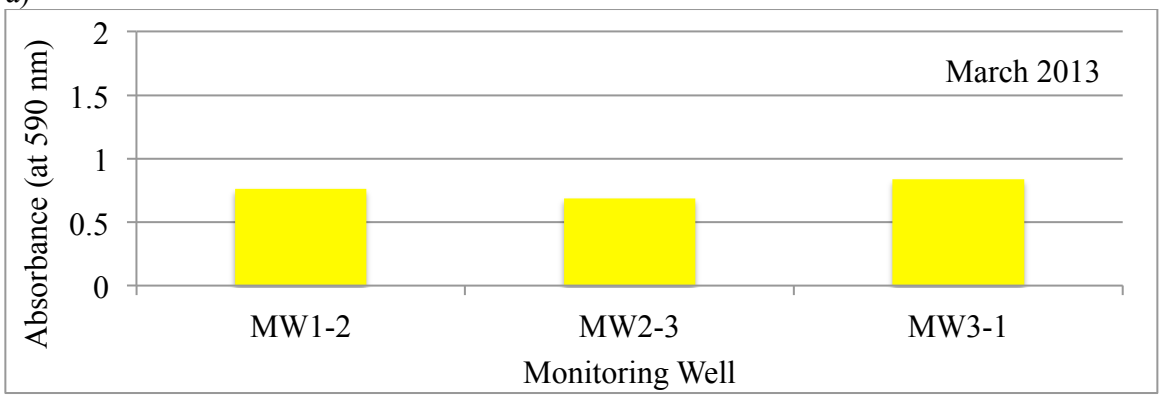
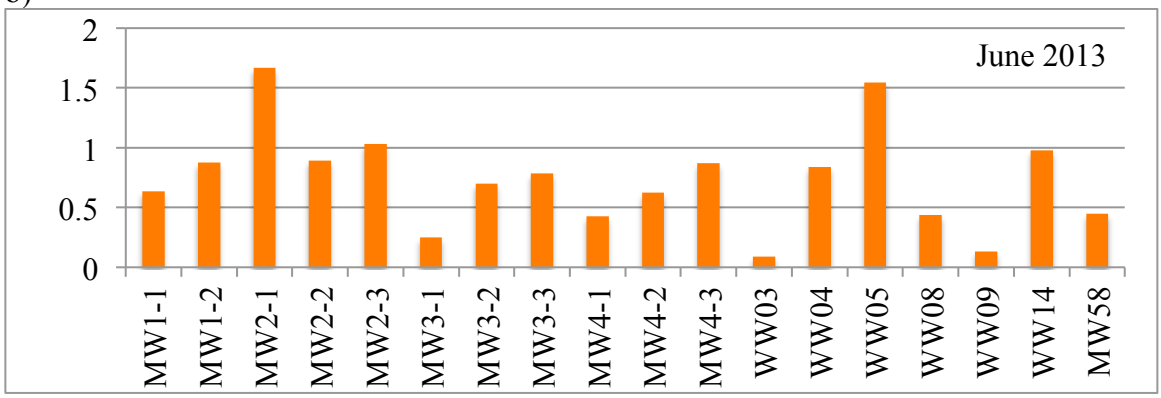


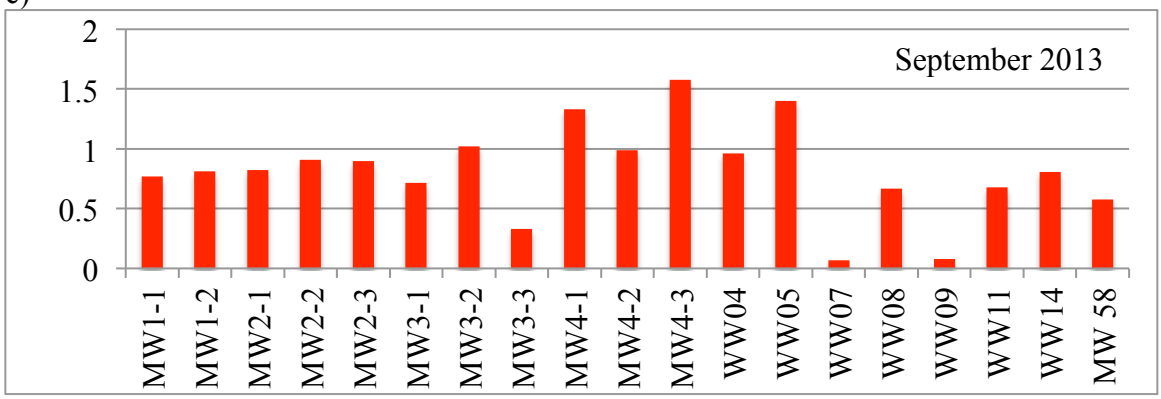
Figure 25. Average Well Color Development for March through December 2013 (a-d): Longhorn

(Figure 25 continued)

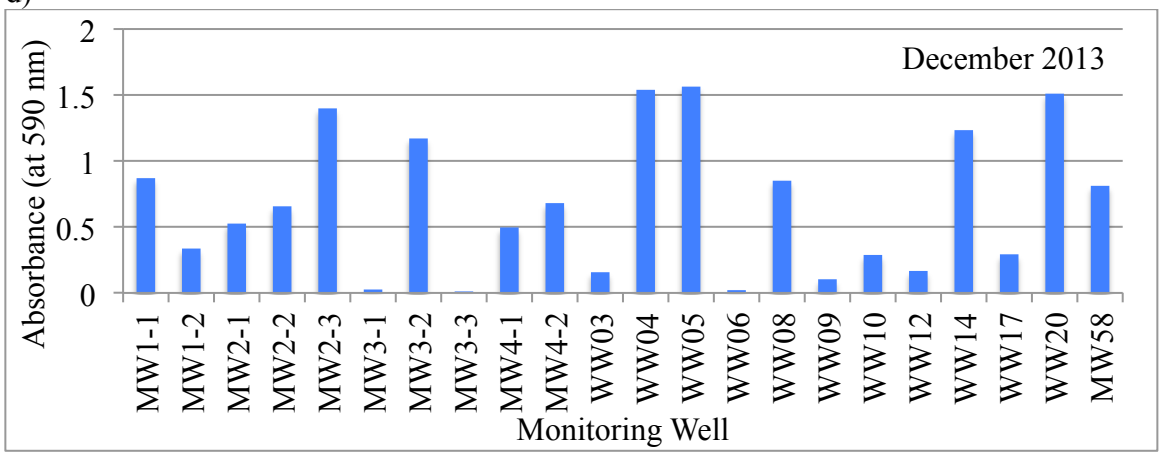
b)



c)



d)



4.2.5 Functional Diversity

In September 2013, WW07 and WW09 were very low in diversity as seen in Figure 26. Overall, the September samples look better than they did in June. The samples have more microbial diversity, and they have higher microbial numbers. In December

2013, WW20 has the largest percent functional diversity. Figure 27 shows the temporal changes in percent functional diversity for a few select monitoring wells.

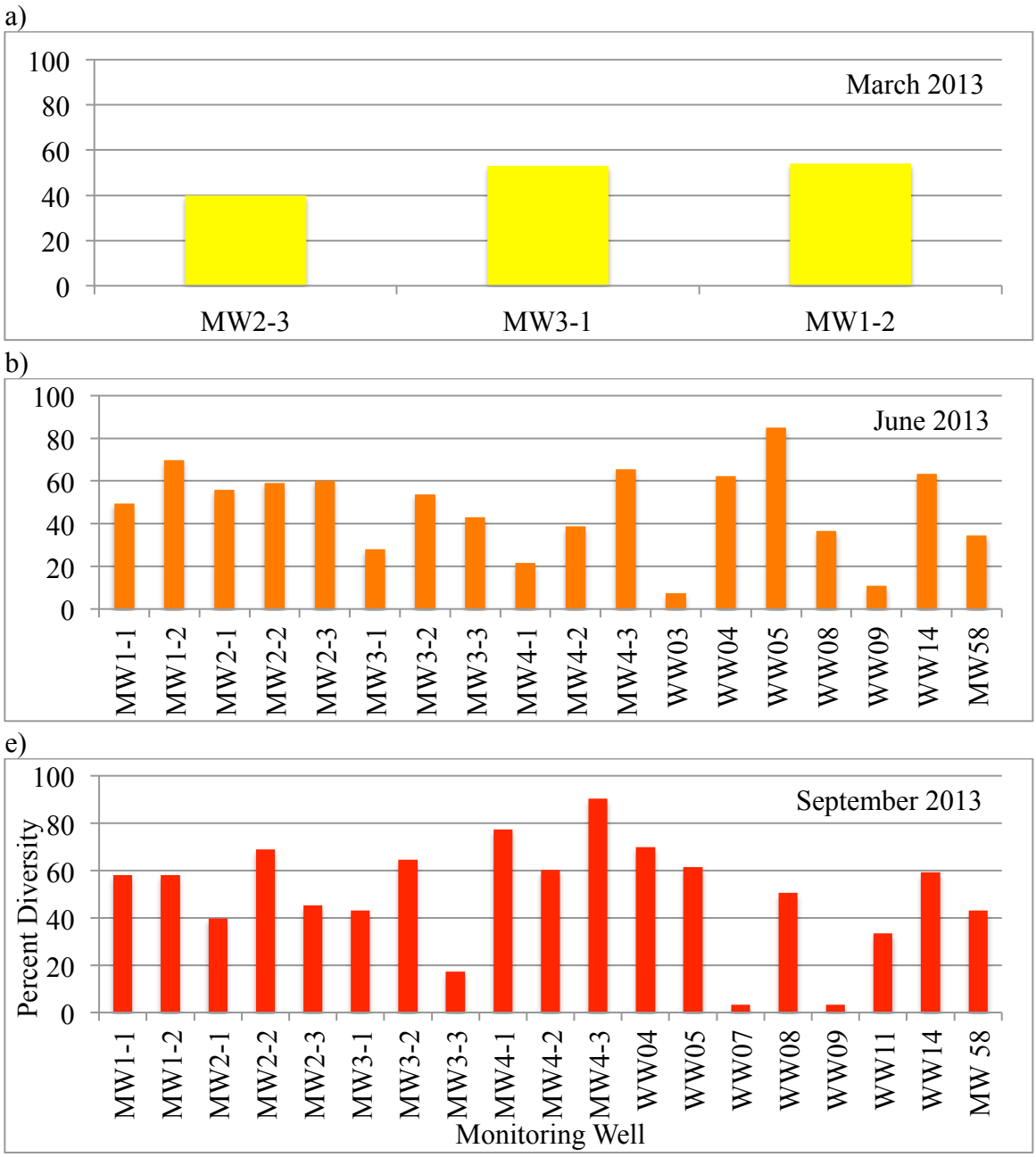
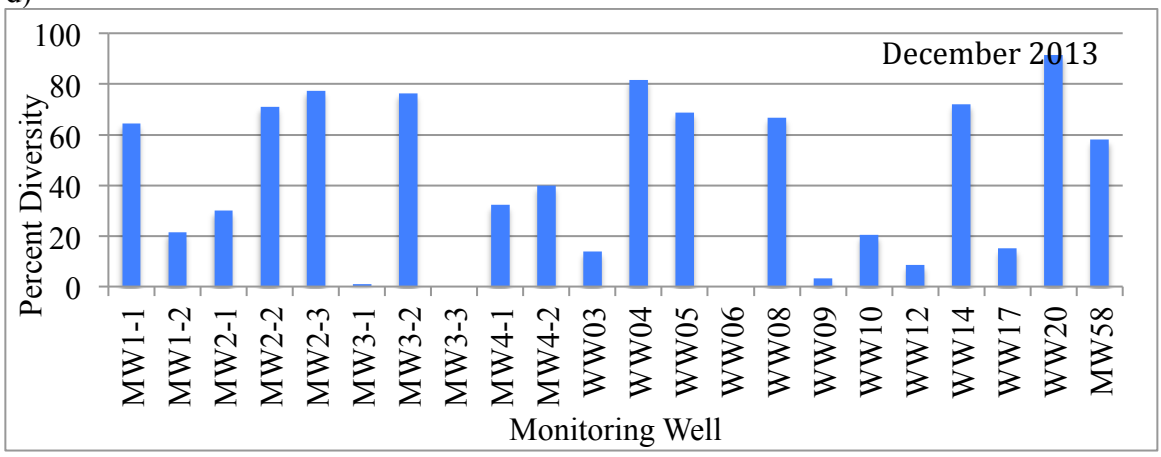


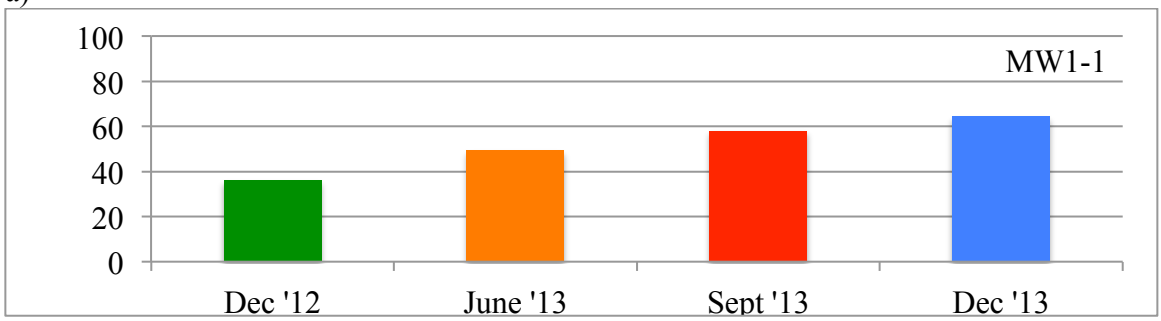
Figure 26. Percent Functional Diversity for March through December 2013 (a-d): Longhorn

(Figure 26 continued)

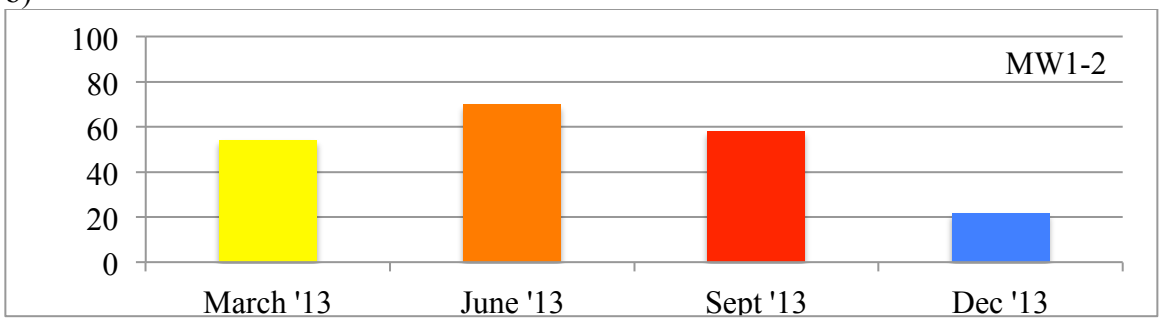
d)



a)



b)



c)

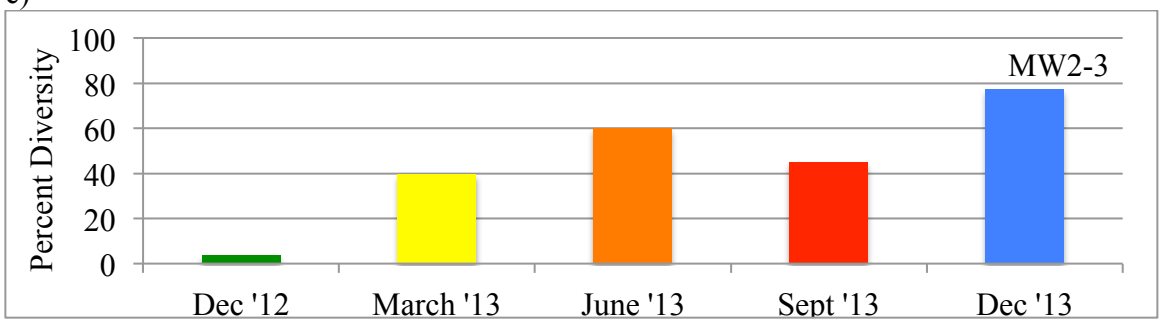
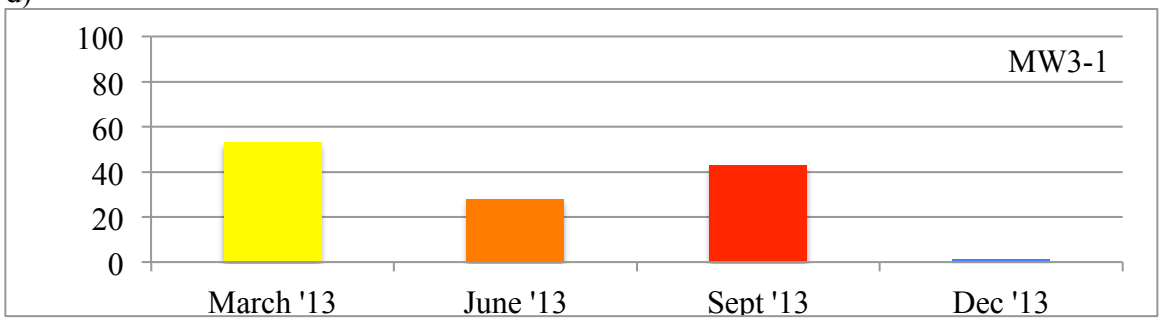


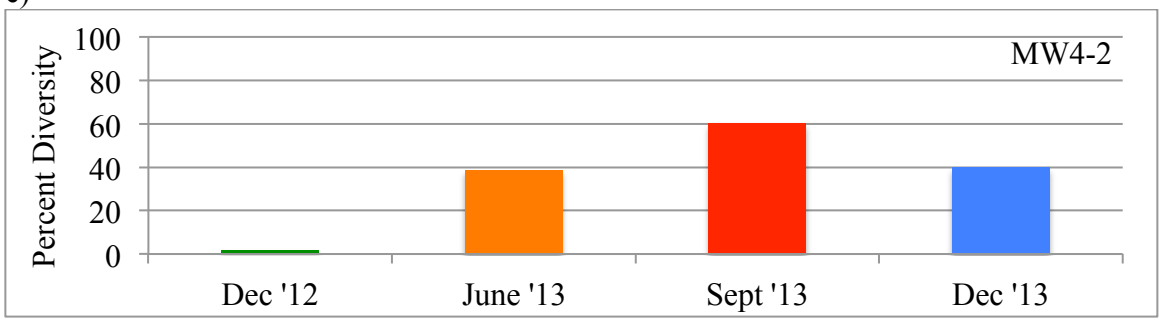
Figure 27. Temporal Change in Functional Diversity for Samples a) MW1-1, b) MW1-2, c) MW2-3, d) MW3-1 and e) MW4-2: Longhorn

(Figure 27 continued)

d)



e)



MW1-1 and MW2-3 saw an increase in functional diversity in December 2013. MW2-2, MW3-1 and MW4-2 saw a decrease in functional diversity in December 2013. This difference could be due to several different variables that are preventing the bio plug microbial populations from reaching the monitoring wells, such as low permeability and low flow rates around the bio plugs. It should be noted that December 2012 samples were analyzed at 450 nm not 590 nm.

4.2.6 Shannon-Weaver Index

In Figure 28, most of the wells have high values meaning substrate usage is diverse and equally distributed. The Shannon-Weaver index and percent functional diversity results are proportionally similar.

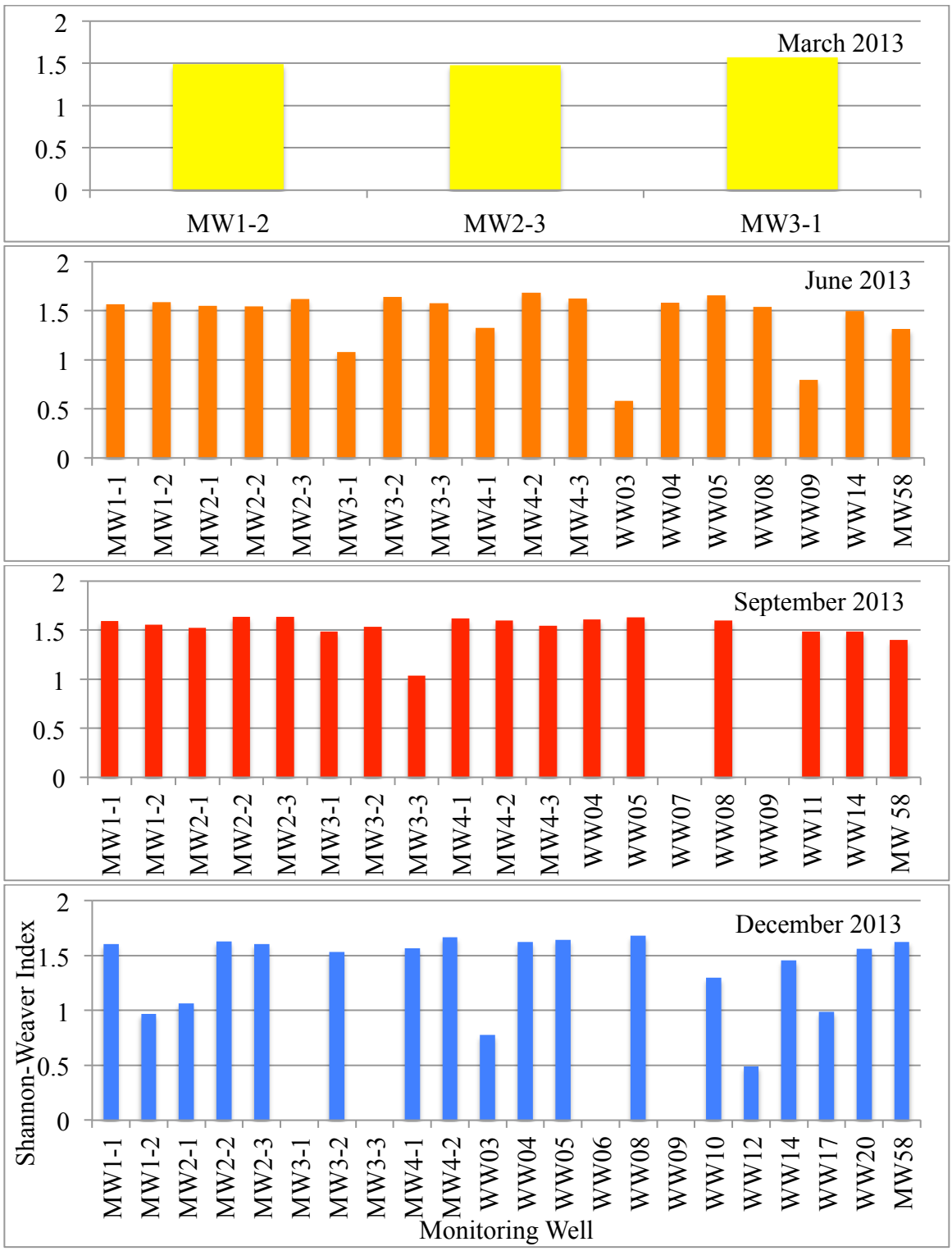


Figure 28. Shannon-Weaver Index for March 2013 through December 2013: Longhorn

4.2.7 Variation in EcoPlate™ Replicates

Figure 29 shows the variation between the replicates on the EcoPlates™. In September 2013, sample WW09 did not have any replicate variation in the EcoPlates™, but it also did not have any diversity as it only used pyruvic acid methyl ester as its carbon source. In December 2013, samples WW06 and MW3-3 did not have any variation between replicates, but they also showed negligible growth on the EcoPlates™.

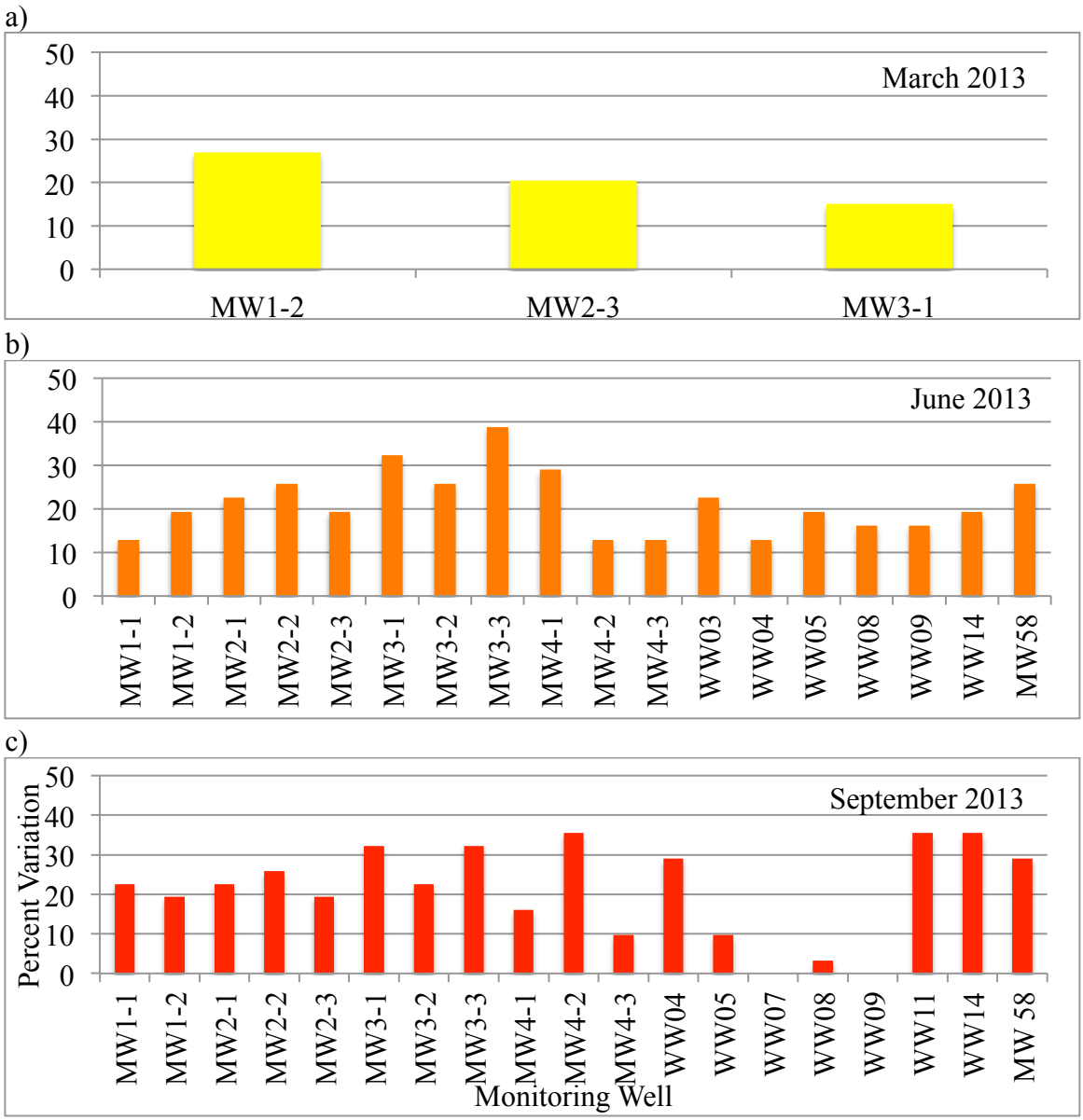
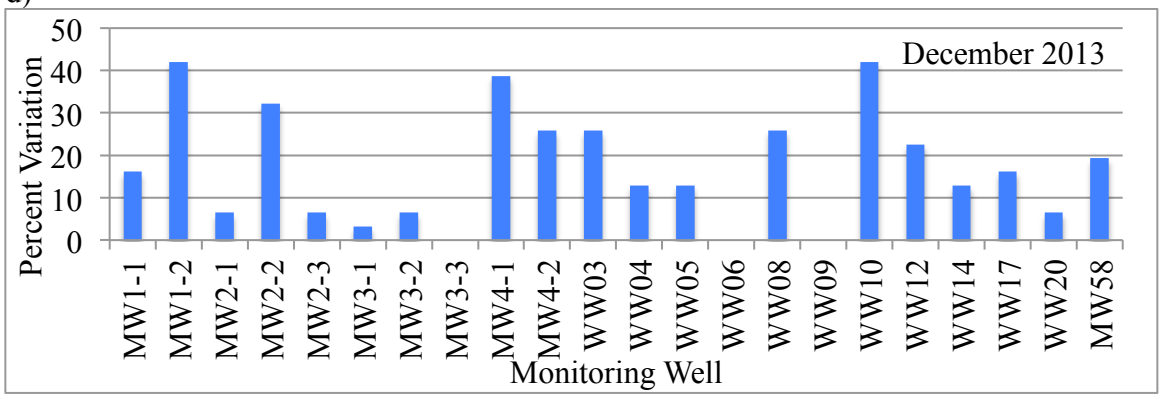


Figure 29. Percent Variation in Replicates on each EcoPlate™ for March 2013 through December 2013 (a-d): Longhorn

(Figure 29 continued)

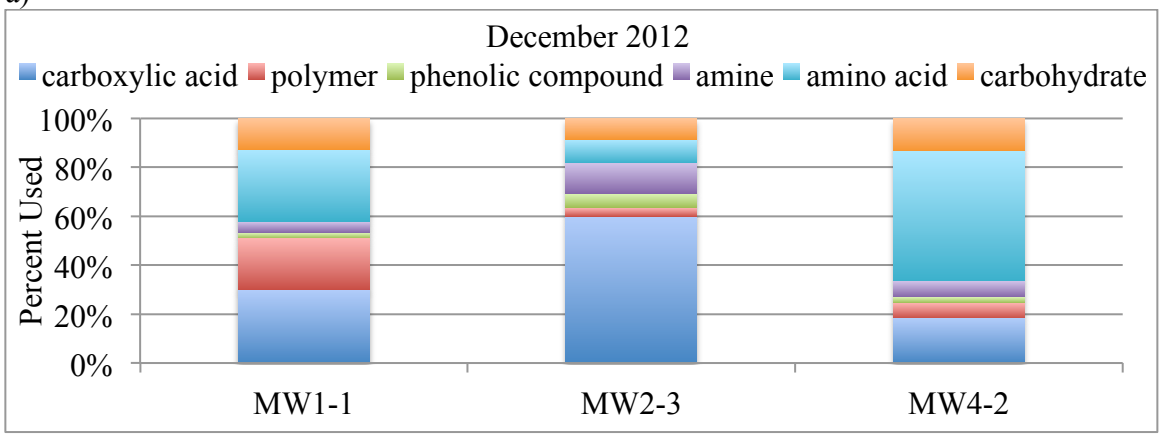
d)



4.2.8 Carbon Substrates Used

In Figure 30, the carbon substrate utilization graphs show the diversity in the microbes present based on the type of carbon used as the energy source.

a)



b)

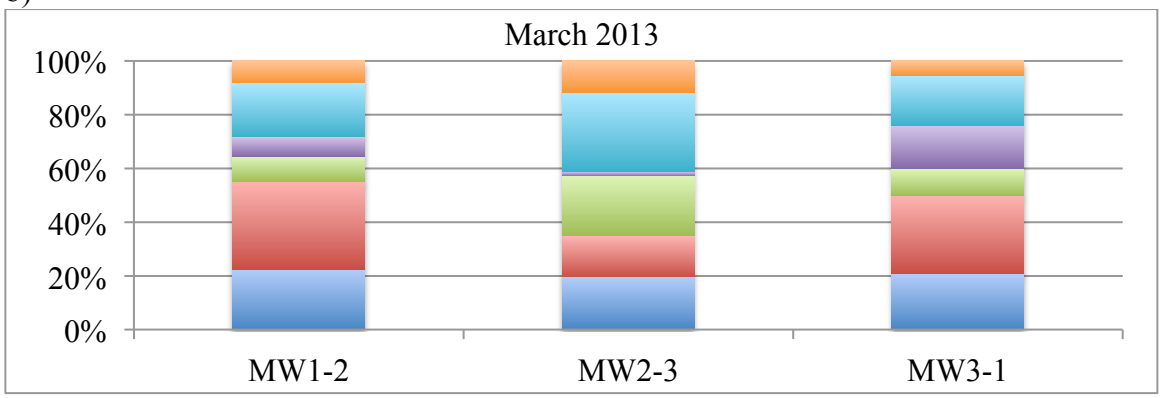
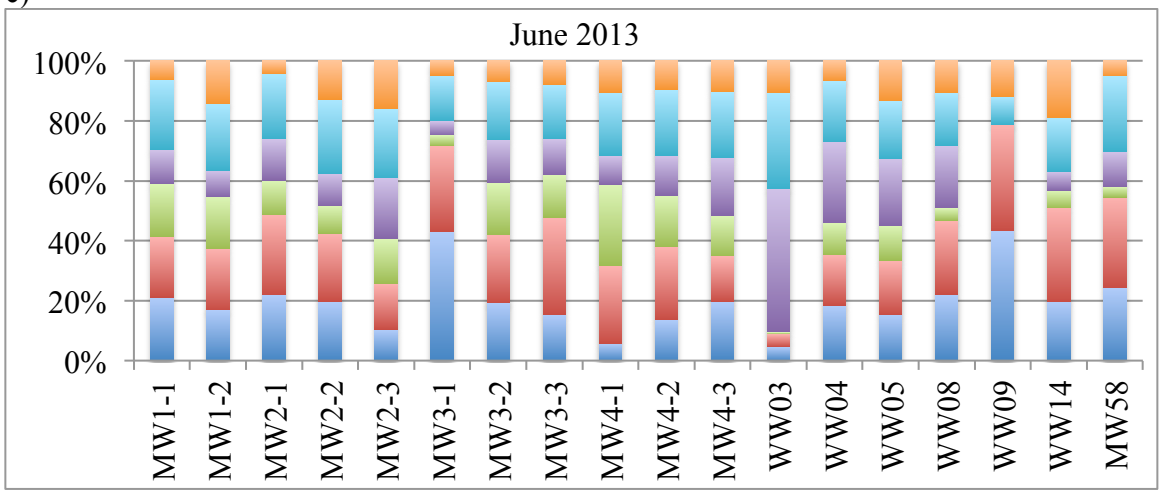


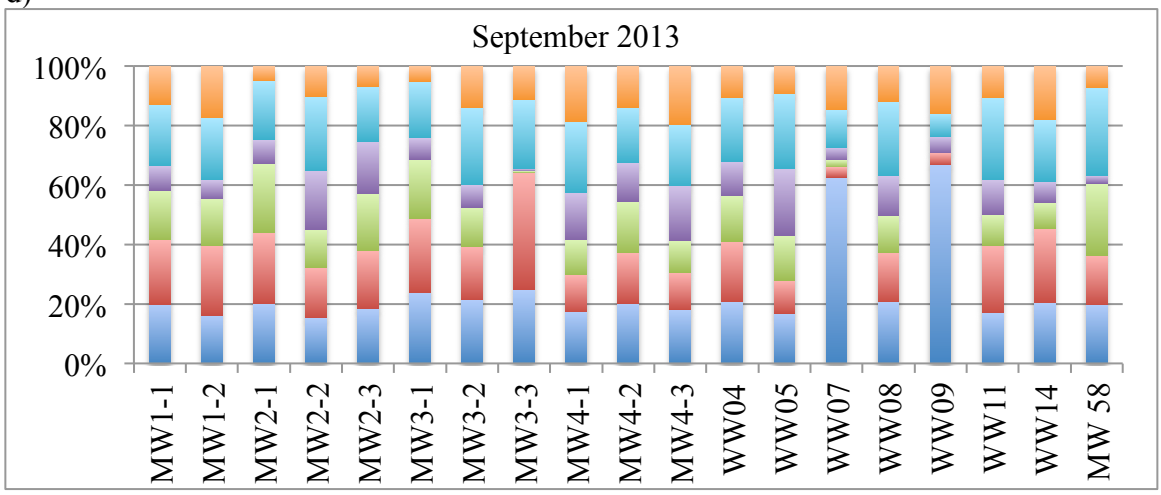
Figure 30. Percent of Carbon Substrates Used from December 2012 to December 2013 (a-e): Longhorn

(Figure 30 continued)

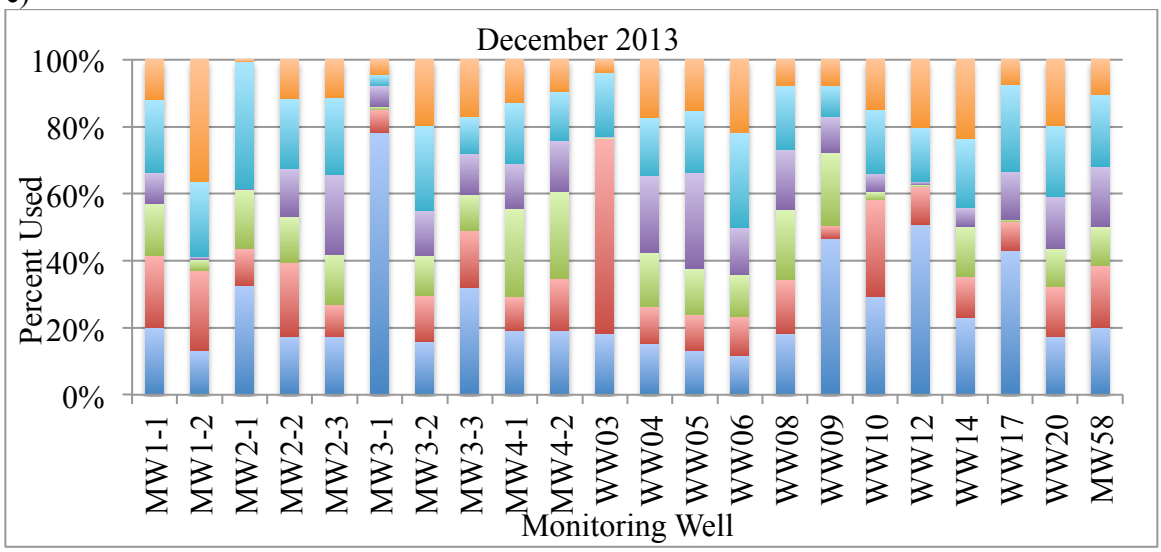
c)



d)



e)



In December 2012, there is a large difference in the types of microbes present among the three wells analyzed, MW2-3, MW4-2 and MW1-1. In March 2013, the carbon substrate utilization became more evenly distributed. In September 2013, the substrate usage became even more evenly distributed; although, three samples were inconsistent with the rest. Monitoring well WW07 and WW09 used carboxylic acids more than 60% of the time and MW3-3 did not use any phenols. In December 2013, some samples became more evenly distributed while others less. For example, MW3-3 increased in phenolic usage from September 2012 to December 2013 while MW3-1 was overwhelmingly dominated by carboxylic acid usage.

Figures 27 and 31 can be compared because a high percent functional diversity should mean more diversity in the substrate usage. MW1-1 had a steady increase in diversity. There was a large increase in evenness from December 2012 until June 2013 then, from June 2013 to December 2013 the evenness was similar. MW1-2 increased in diversity from March 2013 until June 2012 and then there was a steady decrease. This was perfectly reflected in the substrate utilization. MW2-3 had a steady increase in diversity except for a slight decrease in September 2013. The substrate evenness increased from December 2012 until June 2013 and from June 2013 until December 2013 the distribution was similar. MW3-1 had a steady decrease in diversity except for an increase during September 2013. The substrate utilization perfectly matched this. MW4-2 had an increase in diversity from December 2012 until September 2013 then, there was a decrease for December 2013. This was also reflected in the substrate utilization, but the magnitude of the decrease in diversity for December 2013 was not reflected in the

substrate utilization. There was only a slight increase in phenolic compound usage and a decrease in carbohydrate usage.

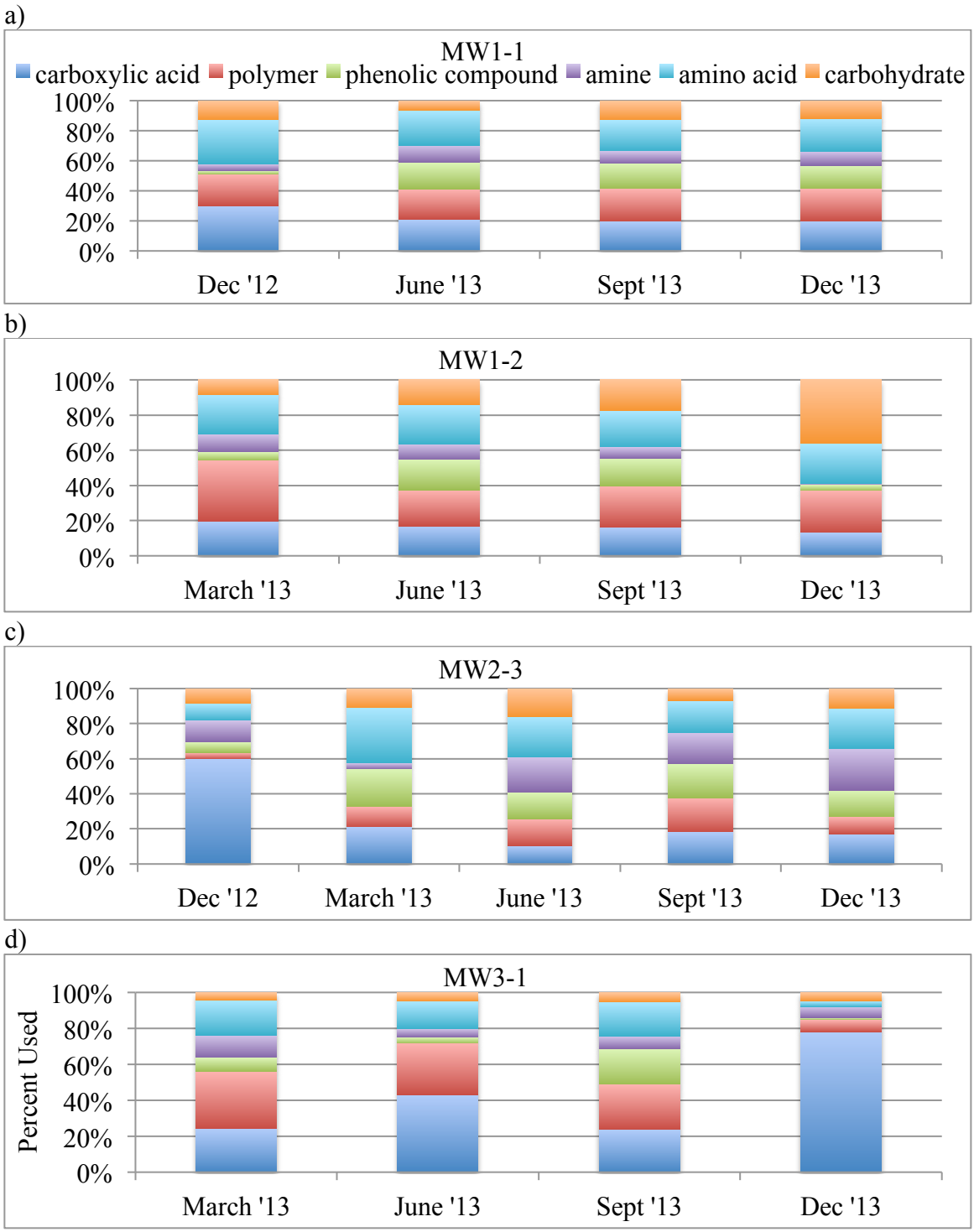
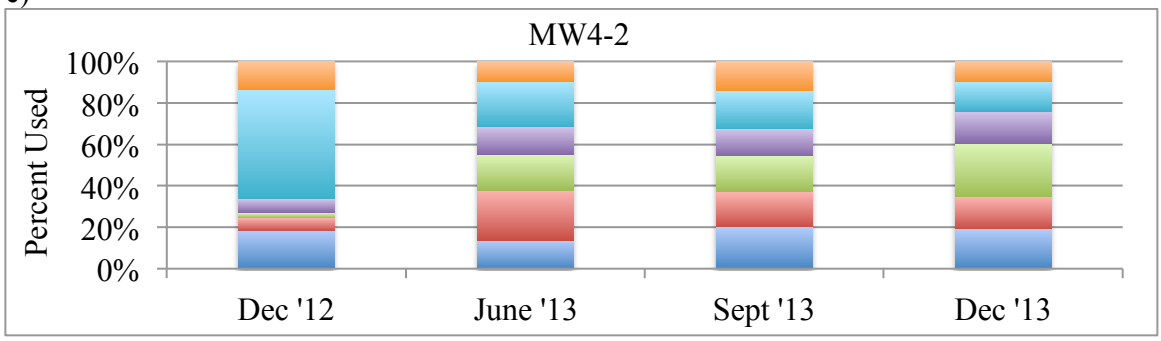


Figure 31. Temporal Change in Carbon Substrates Used for Samples a) MW1-1, b) MW1-2, c) MW2-3, d) MW3-1, and e) MW4-2: Longhorn

(Figure 31 continued)

e)



4.2.9 Species Identification

In September 2013, the following water samples were analyzed and identified as the following species: MW4-1 as *Pseudomonas aeruginosa,* MW1-2 as *Pseudomonas aeruginosa*, and WW04 as *Achromobacter piechaudii* and *Pseudomonas stutzeri*. In June 2013, WW09 was identified as being *Pseudomonas mendocina* and *Pseudomonas stutzeri.* In December 2013, sample WW08 was identified as being *Pseudomonas viridilivida*, while WW09 was identified as having *Pseudomonas nitroreducens/azelaica* and *Pseudomonas mendocina*. The bio plugs were inoculated with *Acinetobacter haemolyticus*, *Pseudomonas aeruginosa*, and *Pseudomonas stutzeri*. Theses species have been found in the groundwater from the monitoring wells at several sampling events. This means the species have successfully moved through the soil.

4.2.10 Acute Toxicity Test

MicroTox® acute toxicity tests were run on December 2013 samples MW58, WW04 and WW12. Tables 9 through 11 show results at time zero before samples were added and 15 minutes after samples were added. The light intensity, I, is measured in absolute light units. Adding the samples barely had an effect on the light intensity. Therefore, the samples are not toxic.

Table 9. Toxicity of MW58: Longhorn

Sample Concentration (mg/L)	I_0	I_{15}
0	93	81
5.63	72	68
11.25	89	84
22.5	99	93
45	66	62

Table 10. Toxicity of WW04: Longhorn

Sample Concentration (mg/L)	I_0	I_{15}
0	91	77
5.63	86	73
11.25	84	70
22.5	75	63
45	72	60

Table 11. Toxicity of WW12: Longhorn

Sample Concentration (mg/L)	I_0	I_{15}
0	83	70
5.63	92	79
11.25	82	72
22.5	92	78
45	89	75

4.2.11 Bio Plug Immobilized Biomass

The bio plugs used to inoculate the site were analyzed in April 2013 for their microbial community structure, so that their results could be compared with the monitoring well results. Bio plug 40-7 is located right next to MW 4 cluster (meaning MW4-1, 4-2 and 4-3). Bio plug 39-4 is also located near the MW 4 cluster. Bio plug 36-6 is near WW04. Bio plug 19-3 is about equidistant from WW03, WW05, WW06, MW 2 cluster, MW 3 cluster, and MW58. Bio plug 29-5 is right next to WW03. Bio plug 27-2 is also near WW03.

Over time, the monitoring wells have come to reflect the bio plugs. In Figure 32, the carbon substrate usage is very well balanced among the substrate guilds. The highest

percent functional diversity was 91 for WW20 in December 2013, as seen in Figure 33. This was also the highest value for the monitoring wells. In Figure 34, the highest variation is lower than the highest variation in the monitoring wells and the lowest values are higher than the lowest values in the monitoring wells, as seen in Figure 29. The AWCD values were all higher than one, which is not the case in the monitoring wells, as seen in Figures 25 and 35. Several of the monitoring wells have Shannon-Weaver indexes over 1.5, as seen in Figure 36. This is not the case with the monitoring wells from Figure 28. The microbial species identified include *Acinetobacter haemolyticus* from 36-6, 29-5, and 40-7, *Pseudomonas aeruginosa* from 27-3, and *Pseudomonas stutzeri* from 29-5, and 40-7.

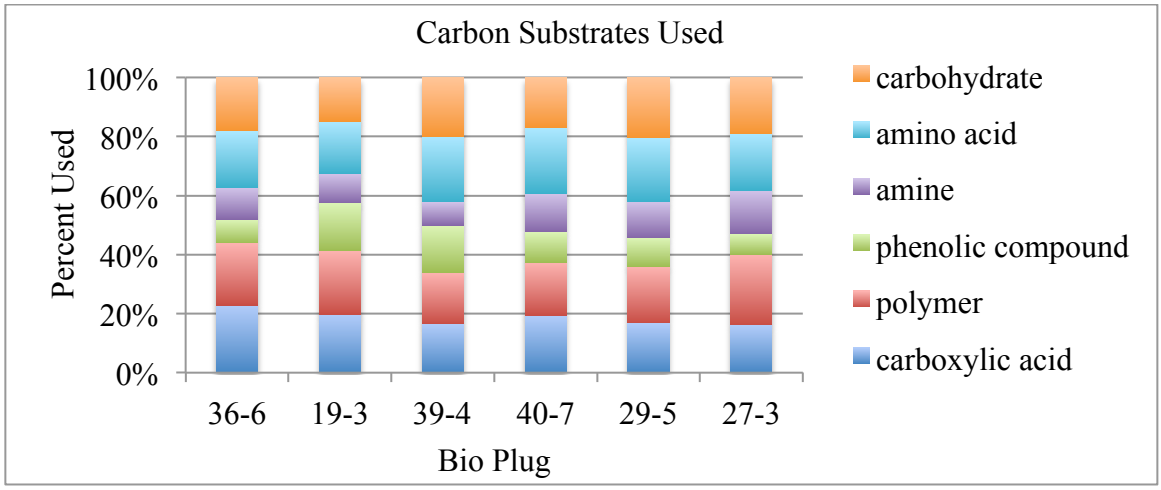


Figure 32. Percent Carbon Substrates Used for Bio Plugs: Longhorn

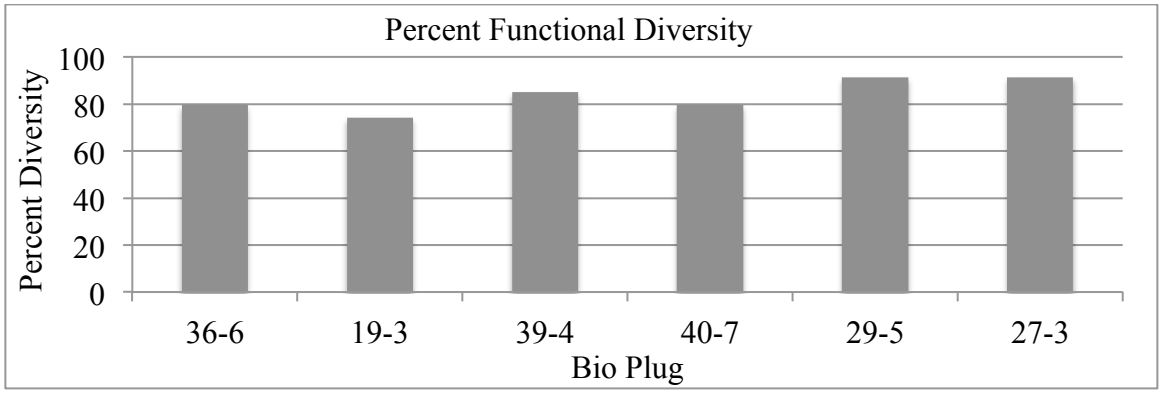


Figure 33. Percent Functional Diversity of Bio Plugs: Longhorn

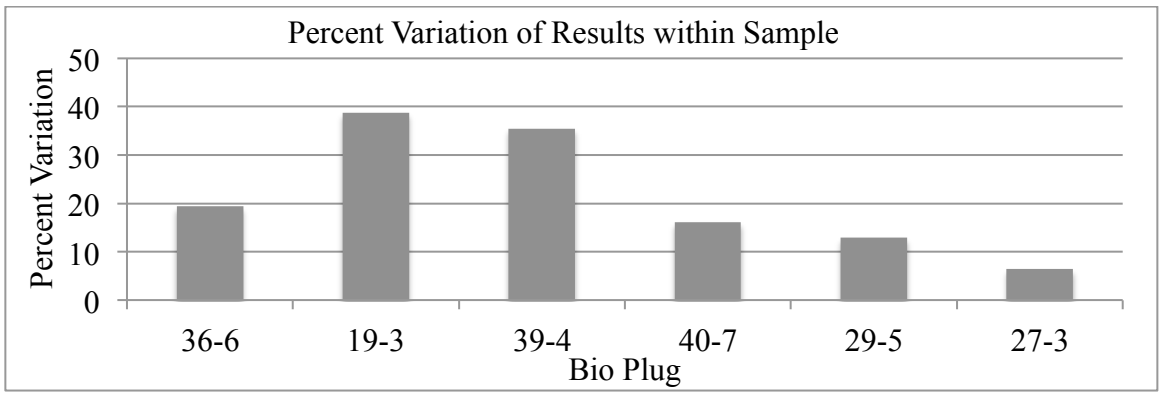


Figure 34. Percent Variation of Results within Sample of Bio Plugs: Longhorn

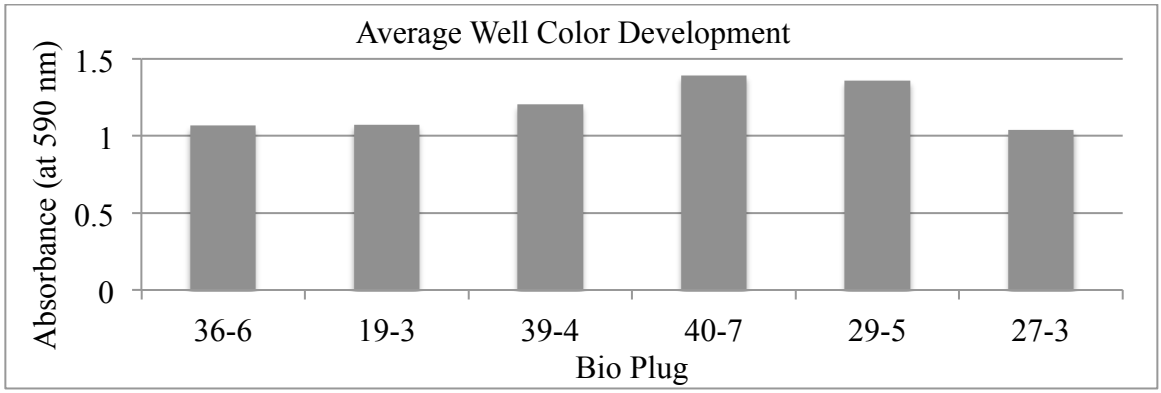


Figure 35. Average Well Color Development of Bio Plugs: Longhorn

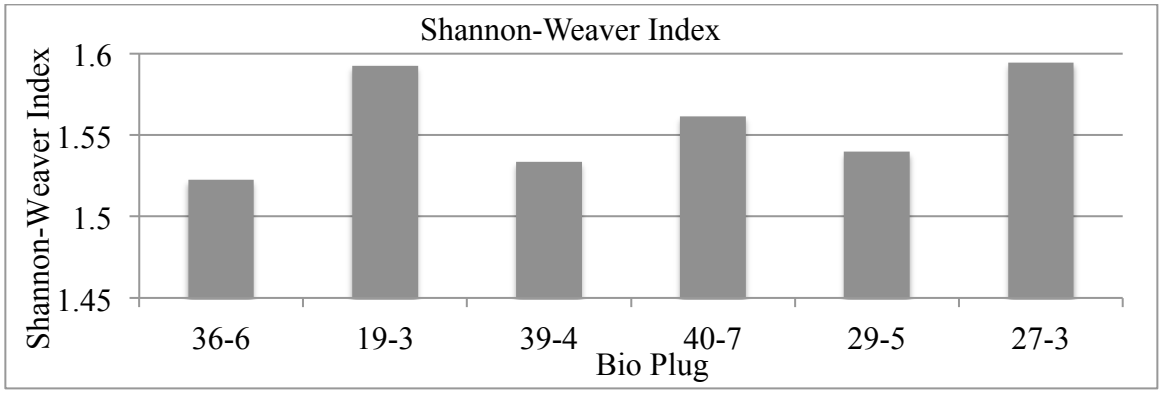


Figure 36. Shannon-Weaver Index of Bio Plugs: Longhorn

4.2.12 Statistical Analysis of Data

An analysis of variance (ANOVA) was done for all the samples using the Shannon-Weaver index. This looks at the variability between sample groups and within sample groups. If the p-value is close to zero, the null hypothesis is rejected. The null hypothesis is that the samples are drawn from populations with the same mean. So, a very

small p-value (less than 0.01) means there is a significant difference between samples. The samples have nine replicates (except the bio plugs which have 3) and are divided into subgroups of monitoring well sites and groups based on the date of sampling. The first calculation compared December 2012 and March 2013 samples. The group p-value is 0.0741 and the subgroup p-value is 0 meaning there is a significant difference between subgroups but not between groups.

An ANOVA was done for the April 2013 bio plug samples. The p-value is 0.7806 meaning the samples are similar. This is expected since the bio plugs were all inoculated with the same bacterial communities.

The ANOVA between the March 2013 samples and April 2013 bio plug samples has a group p-value of 0.1160 and a subgroup p-value of 0.1323 meaning there is no significant difference between the groups or subgroups.

Finally, an analysis of variance was done using the percent functional diversity data. The results were the same. The same conclusions can be drawn for an ANOVA using Shannon-Weaver index and percent functional diversity.

In conclusion, the December 2012 and March 2013 samples on the whole were not significantly different from each other. The bio plug and March 2013 samples on the whole were also not significantly different from each other. The December 2012 samples were different between locations. The March 2013 samples were also different between locations, but this could be only due to an outlier. The April 2013 bio plug samples were the same between locations.

5 SUMMARY AND CONCLUSIONS

5.1 Sunnyvale Site

In the Intermediate Transmissive Zone, the concentrations are on the decline. The decrease is statistically significant overtime with a p-value of 0.00002. The 2B (p=0.40844) and 2nd (p=0.28794) Transmissive Zones have somewhat stable concentrations and do not have statistically significant change overtime. The reason for this lack of change is because the bio plugs are not attached to those zones. As seen in Figure 37, the TCE concentration increases when precipitation is low. A lack of rainfall can influence the groundwater TCE concentrations.

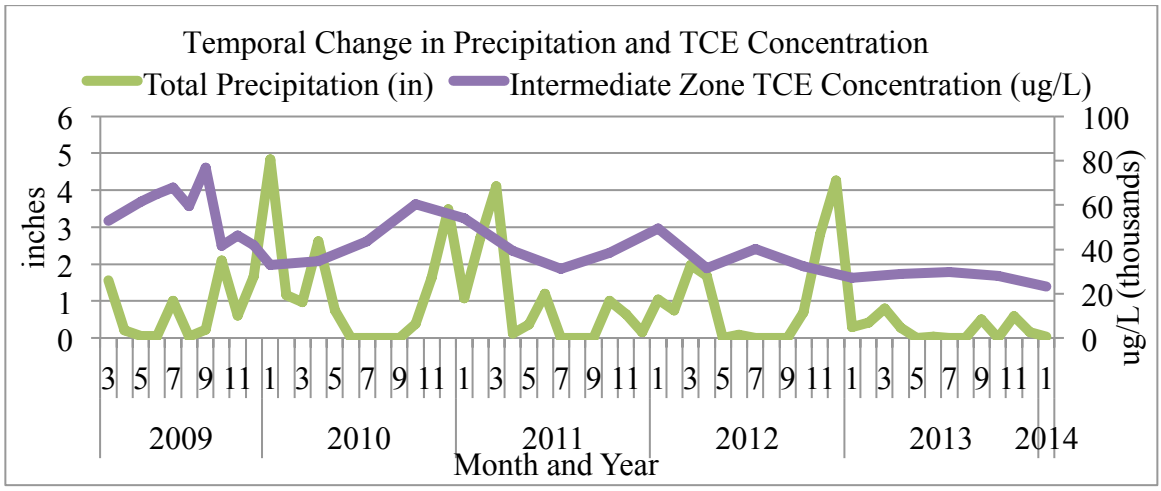


Figure 37. Temporal Change in Precipitation and TCE Concentration from March 2009 to January 2014: Sunnyvale

Over the 17 months of chlorinated aliphatic degrader population counts, the average CFU/ml was 297 in August 2012, 237 in January 2013, 12 in July 2013, and 111 in January 2014. From August 2012 to January 2013 the numbers decrease, from January 2013 to July 2013 the numbers decrease, from July 2013 to January 2014 the numbers increase. These numbers do not seem to follow a seasonal pattern. Over the 6 months of

heterotroph population counts, the average CFU/ml was 222 in July 2013 and 748 in January 2014. This pattern follows that of the chlorinated aliphatic degraders.

Likewise, the EcoPlate™ data followed a similar increasing trend. The average percent functional diversity increased from 1.83% in July 2013 to 9.03% in January 2014. The average AWCD increased from 0.04 in July 2013 to 0.14 in January 2014. The carbon substrate utilization did not show much improvement toward a more even guild distribution, but the low diversity numbers can explain this. The acute toxicity tests indicate that the TCE levels are not high enough to be toxic to *Vibrio fischeri*, the indicator species. The MicroPlates™ identified different species in the monitoring wells at each sampling event. Also, the species present in the bio plug pellets were not identified in the monitoring well samples. The downside to this site's research is that there were only three sampling events. More sampling events will provide stronger data.

5.2 Longhorn Site

The TCE concentrations show a declining trend, but not a statistically significant one (p=0.08598). The largest decline in concentration since July 2012 was in WW08. Figure 38 shows the relationship between precipitation and TCE concentrations. When precipitation was at its highest, the TCE concentration was low.

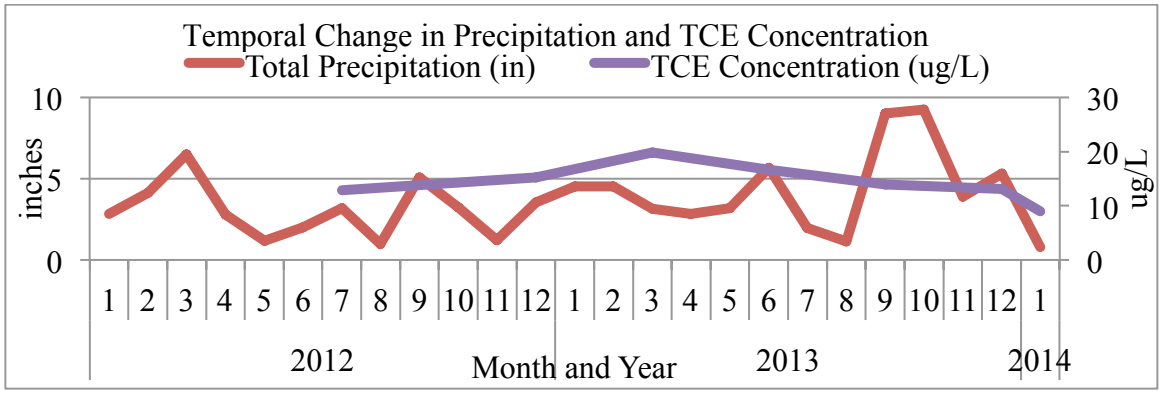


Figure 38. Temporal Change in Precipitation and TCE Concentration from January 2012 to January 2014: Longhorn

Over the 17 months of chlorinated aliphatic degrader population counts, the average CFU/ml was 3145 in July 2012, 84979 in September 2012, 465 in December 2012, 42 in March 2013, 13 in June 2013, 73 in September 2013, and 234 in December 2013. There may be seasonal influence in these numbers. From July 2012 to September 2012 the numbers increase, from September 2012 to December 2012 the numbers decrease, from December 2012 to March 2013 the numbers decrease, from March 2013 to June 2013 the numbers decrease, from June 2013 to September 2013 the numbers increase and from September 2013 to December 2013 the numbers increase. Over the 17 months of heterotroph population counts, the average CFU/ml was 3502 in July 2012, 166595 in September 2012, 39 in December 2012, 3231 in September 2013, and 5226 in December 2013. This pattern follows that of the chlorinated aliphatic degraders.

The relationship among TCE concentration and microbial community assays can be seen in Figures 39 - 41. When TCE concentration is compared with chlorinated aliphatic degrader populations, one can see in inverse relationship. As biomass increases, TCE decreases. June 2013 results are an exception. This could be due to the lack of rainfall as seen in Figure 39.

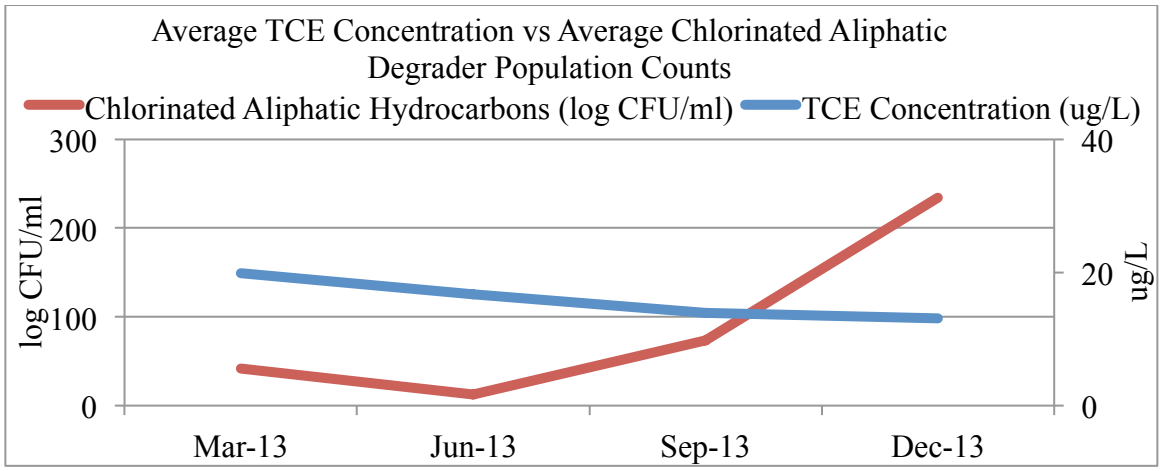


Figure 39. Average TCE Concentration vs Average Chlorinated Aliphatic Hydrocarbon Population Counts: Longhorn

The same relationship is seen in Figure 40 when looking at heterotroph populations. TCE and percent functional diversity also have an inverse relationship (Figure 41).

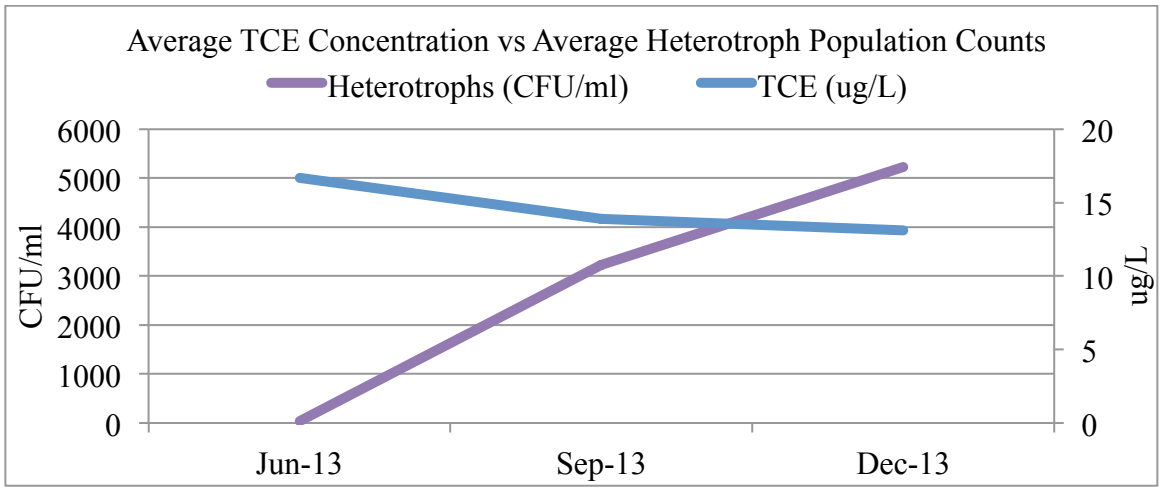


Figure 40. Average TCE Concentration vs Average Heterotroph Population Counts: Longhorn

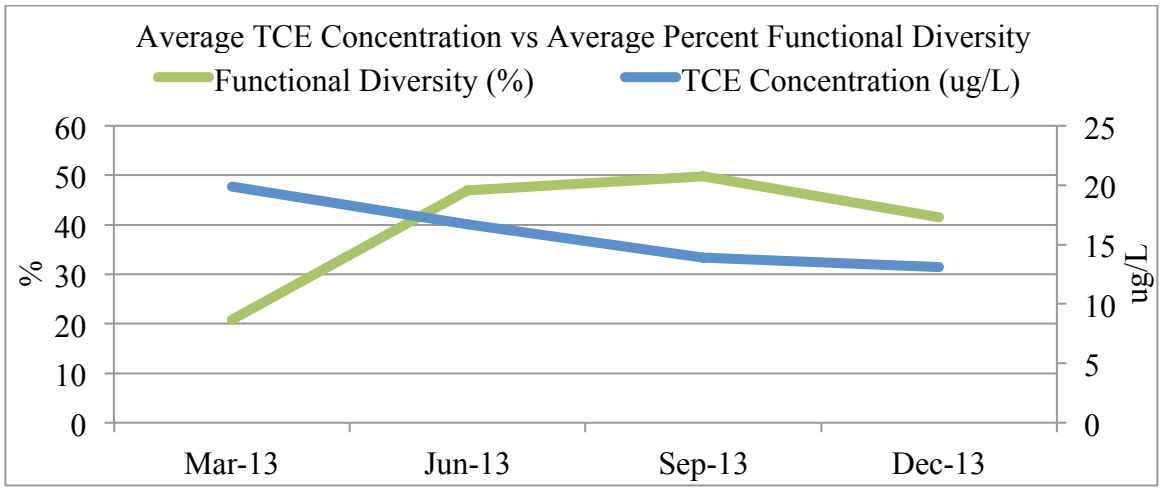


Figure 41. Average TCE Concentration vs Average Percent Functional Diversity: Longhorn

When the assays are compared to each other, a clear relationship cannot be seen. Figure 42 compares percent functional diversity with chlorinated aliphatic degrader population counts. Figure 43 compares percent functional diversity with heterotroph population counts.

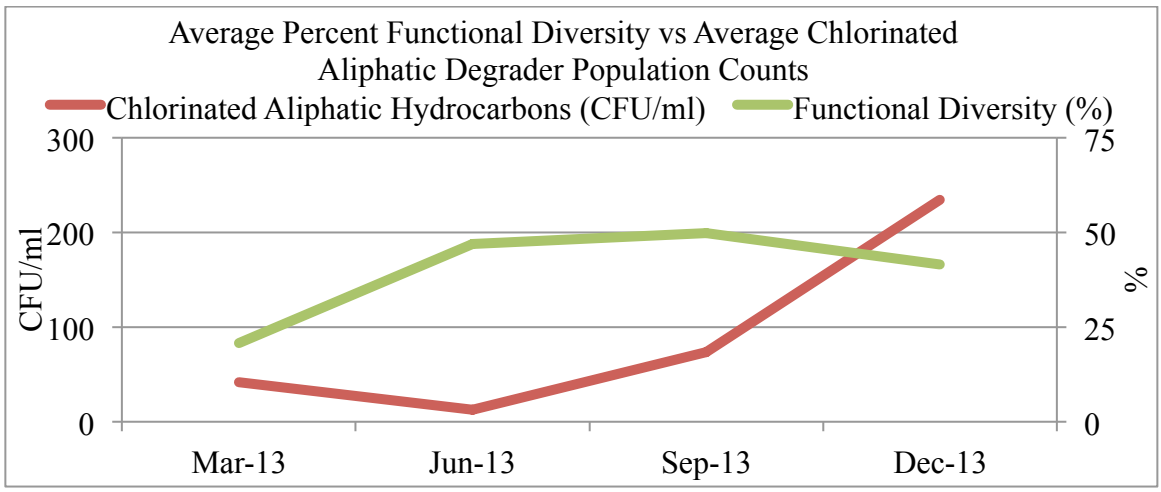


Figure 42. Average Percent Functional Diversity vs Average Chlorinated Aliphatic Hydrocarbon Degrader Population Counts: Longhorn

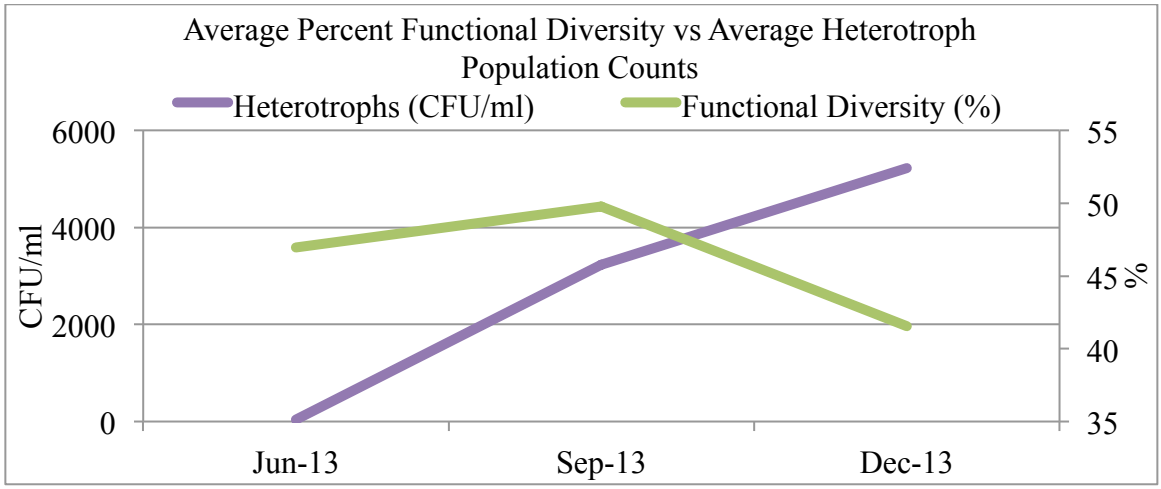


Figure 43. Average Percent Functional Diversity vs Average Heterotroph Population Counts: Longhorn

The acute toxicity tests indicate that the TCE levels are not high enough to be toxic to *Vibrio fischeri*. The species present in the bio plug pellets were identified in the monitoring wells. Some of the species identified show up as being present in more than one monitoring well. The downside to this site's research is that not all of the monitoring wells were sampled during each sampling event, but this could not be helped because the sampling followed U.S. Army protocol.

5.3 Conclusion

The 2014 datasets will help determine whether or not the bioremediation projects were successful. The bio plugs are doing their jobs, as verified by the analysis of the microbial communities. The Shannon-Weaver index, average well color development, functional diversity, population counts, carbon substrate utilization, and VOC concentration results complement each other for the most part. There is a correlation between increased microbial diversity and abundance and decreased VOC concentrations. For our purposes, the Biolog assays were a good illustration of the microbial community structure. Only a few of the inoculated MicroPlates^{TM} had growth to identify when plate counts indicated otherwise. Of those that had growth, only one species with a few colonies appeared. With the proof of high diversity, there should be more than one species to identify. The two sites did not have any microbial species in common. Both sites were inoculated with the same organisms. The suspect *Ochrobactrum* species identified in the Sunnyvale bio plugs is due to taking bio plug pellet samples off the top of the bio plugs and not from deep in the ground. There was no clear relationship between the traditional assays and the new ones. This is due to the fact that the traditional ones use simple sugars and sole carbon substrates and the new ones use specific complex substrates. There is an inverse relationship between TCE concentrations and the traditional assays and TCE concentrations and the new assays. The various tools used for analyzing the microbial communities at these sites all came together and formed a nice package for illustrating said communities.

5.4 Future Research

Traditional methods for assessing *in situ* microbial communities often provide limited information on bioremediation transitional processes in substrate utilization. The goal of this research was to assess new methods for describing the microbial communities found in the groundwater and affected contaminated soils so as to document changes in community structure, population dynamics, substrate utilization and biodegradation of constituents of concern during a site remediation. The Biolog assays are a great way to acquire a qualitative description of the microbial community structure. The 2014 datasets will allow more research to be done to obtain more definitive results. The CoC concentrations should be the ultimate proof of whether or not a site is making progress. Still, the EcoPlates™ should be used to accompany bioremediation strategies. They are inexpensive and very easy to use. More research should be done to analyze the accuracy of these methods to know how much confidence can be put in them. There are many variables that can influence the accuracy such as human error, differences in *in situ* and *in vitro* conditions, and time from when the samples were pulled until they reached the lab. While microbes inoculated at the sites are chosen for their stability after storage, some species in the monitoring well groundwater samples may not survive long enough to be analyzed *in vitro*. The EcoPlate™ results, population counts, and CoC concentrations agree with each other and show the same trends. There may not be a perfect solution or strategy. For example, PCR is a more detailed, thorough approach, but it may cost more money and still not be feasible. Ideally, microbial populations from contaminated soils should be compared with those in non-contaminated soils to see any difference between the original communities and those in the presence of contamination. For this research,

the EcoPlates™ showed a temporal increase in population counts and diversity, but a spatial comparison would be a beneficial addition. Also, due to the high cost of soil sampling, we do not have soil samples to compare and analyze. We achieved our goal of assessing traditional and new assays that describe the microbial communities found in the groundwater and affected contaminated soils. We also answered our hypothesis. Changes in community structure, population dynamics, substrate utilization and biodegradation of constituents of concern can be successfully described by EcoPlate™ and MicroPlate™ (Biolog, LLC) assays, population counts, and MicroTox® acute toxicity tests.

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APPENDIX

A. Biolog Microplate™ Instructions for Use

GEN III MicroPlate™

Instructions for Use

Not for human in vitro diagnostic use

BIOLOG

21124 Cabot Blvd.

Hayward, CA 94545

TEL 510-785-2564, FAX 510-782-4639

ORDERS 1-800-284-4949

www.biolog.com

Part# 00P 185, Rev A MAR 2008

INSTRUCTIONS FOR USE OF THE BIOLOG GEN III MICROPLATE™

Intended Use

The GEN III MicroPlate™ test panel provides a standardized micromethod using 94 biochemical tests to profile and identify a broad range of Gram-negative and Gram-positive bacteria[1,2]. Biolog's Microbial Identification Systems software (e.g.OmniLog® Data Collection) is used to identify the bacterium from its phenotypic pattern in the GEN III MicroPlate.

Description

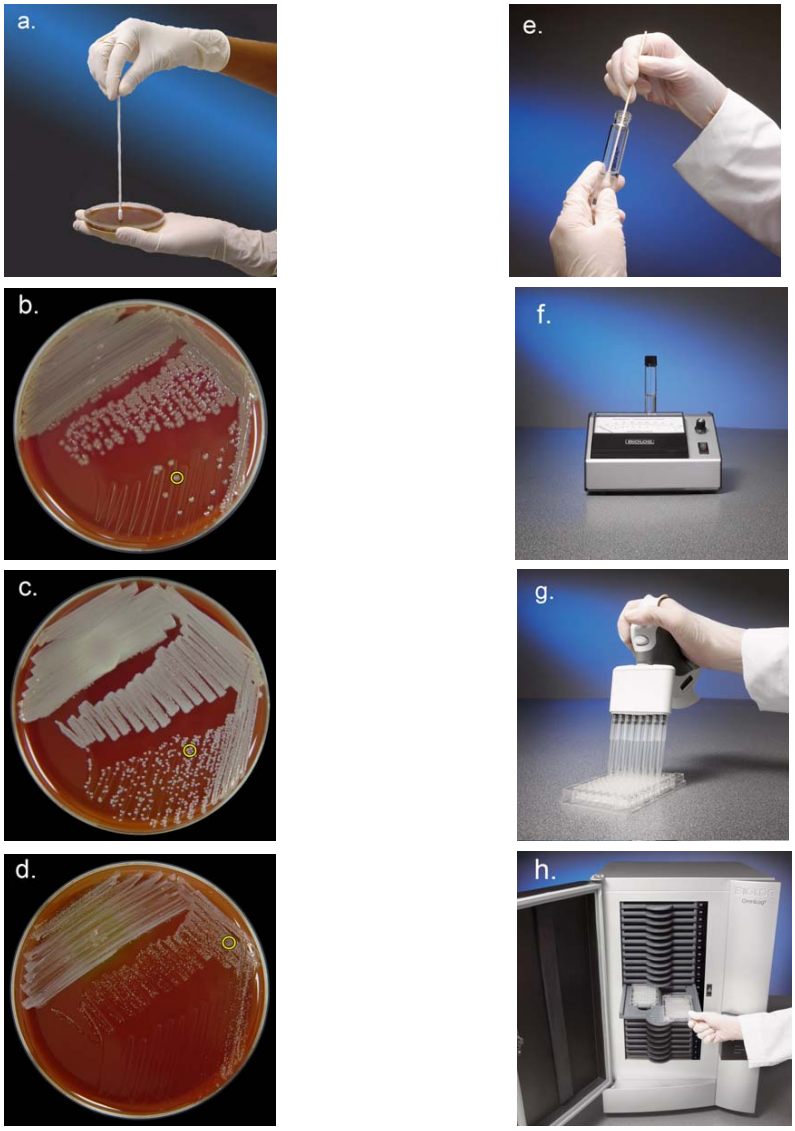
- The Biolog GEN III MicroPlate analyzes a microorganism in 94 phenotypic tests: 71 carbon source utilization assays (**Figure 1, columns 1-9**) and 23 chemical sensitivity assays (**Figure 1, columns 10-12**). The test panel provides a **"Phenotypic Fingerprint"** of the microorganism that can be used to identify it at the species level.
- All necessary nutrients and biochemicals are prefilled and dried into the 96 wells of the MicroPlate. Tetrazolium redox dyes are used to colorimetrically indicate utilization of the carbon sources or resistance to inhibitory chemicals.
- Testing is performed very simply, as shown in **Figure 2**. The isolate to be identified is grown on agar medium and then suspended in a special "gelling" inoculating fluid[3] (IF) at the recommended cell density. Then the cell suspension is inoculated into the GEN III MicroPlate, 100 µl per well, and the MicroPlate is incubated to allow the phenotypic fingerprint to form. All of the wells start out colorless when inoculated. During incubation there is increased respiration in the wells where cells can utilize a carbon source and/or grow. Increased respiration causes reduction of the tetrazolium redox dye, forming a purple color. Negative wells remain colorless, as does the negative control well (A-1) with no carbon source. There is also a positive control well (A-10) used as a reference for the chemical sensitivity assays in columns 10-12. After incubation, the phenotypic fingerprint of purple wells is compared to Biolog's extensive species library. If a match is found, a species level identification of the isolate is made.

Figure 1. Layout of assays in the MicroPlate

GEN III MicroPlate ™

A1 Negative Control	A2 Dextrin	A3 D-Maltose	A4 D-Trehalose	A5 D-Cellobiose	A6 Gentiobiose	A7 Sucrose	A8 D-Turanose	A9 Stachyose	A10 Positive Control	A11 pH 6	A12 pH 5
B1 D-Raffinose	B2 α-D-Lactose	B3 D-Melibiose	B4 β-Methyl-D-Glucoside	B5 D-Salicin	B6 N-Acetyl-D-Glucosamine	B7 N-Acetyl-β-D-Mannosamine	B8 N-Acetyl-D-Galactosamine	B9 N-Acetyl Neuraminic Acid	B10 1% NaCl	B11 4% NaCl	B12 8% NaCl
C1 α-D-Glucose	C2 D-Mannose	C3 D-Fructose	C4 D-Galactose	C5 3-Methyl Glucose	C6 D-Fucose	C7 L-Fucose	C8 L-Rhamnose	C9 Inosine	C10 1% Sodium Lactate	C11 Fusidic Acid	C12 D-Serine
D1 D-Sorbitol	D2 D-Mannitol	D3 D-Arabitol	D4 myo-Inositol	D5 Glycerol	D6 D-Glucose-6-PO4	D7 D-Fructose-6-PO4	D8 D-Aspartic Acid	D9 D-Serine	D10 Troleandomycin	D11 Rifamycin SV	D12 Minocycline
E1 Gelatin	E2 Glycyl-L-Proline	E3 L-Alanine	E4 L-Arginine	E5 L-Aspartic Acid	E6 L-Glutamic Acid	E7 L-Histidine	E8 L-Pyroglutamic Acid	E9 L-Serine	E10 Lincomycin	E11 Guanidine HCl	E12 Niaproof 4
F1 Pectin	F2 D-Galacturonic Acid	F3 L-Galactonic Acid Lactone	F4 D-Gluconic Acid	F5 D-Glucuronic Acid	F6 Glucuronamide	F7 Mucic Acid	F8 Quinic Acid	F9 D-Saccharic Acid	F10 Vancomycin	F11 Tetrazolium Violet	F12 Tetrazolium Blue
G1 p-Hydroxy-Phenylacetic Acid	G2 Methyl Pyruvate	G3 D-Lactic Acid Methyl Ester	G4 L-Lactic Acid	G5 Citric Acid	G6 α-Keto-Glutaric Acid	G7 D-Malic Acid	G8 L-Malic Acid	G9 Bromo-Succinic Acid	G10 Nalidixic Acid	G11 Lithium Chloride	G12 Potassium Tellurite
H1 Tween 40	H2 γ-Amino-Butyric Acid	H3 α-Hydroxy-Butyric Acid	H4 β-Hydroxy-D,L-Butyric Acid	H5 α-Keto-Butyric Acid	H6 Acetoacetic Acid	H7 Propionic Acid	H8 Acetic Acid	H9 Formic Acid	H10 Aztreonam	H11 Sodium Butyrate	H12 Sodium Bromate

Figure 2. Steps in the testing protocol



Materials Provided

- **MicroPlates:** Catalog No.1030- Biolog GEN III MicroPlates (10/box).
- **On receipt, inspect** foil pouches and MicroPlates for damage in shipping. To maintain the full shelf life, the foil pouched MicroPlates must be **stored at 2-8° C**. **The expiration date** is printed on each pouch. Do not use the MicroPlates after the expiration date.

Materials Not Provided

- **Agar Culture Media**: Catalog No.71102-BUG Agar with 5% sheep blood (**BUG+B**); Catalog No. Bio-M1012- **Chocolate Agar**; Catalog No.70101- Biolog Dehydrated Growth Agar, 500 gm (**BUG Agar**).
- **Inoculating Fluid:** Catalog No.72401- **IF-A,** Catalog No.72402- **IF-B,** Catalog No. 72403**- IF-C**.
- **Inoculatorz™**: Catalog No.3321- Sterile disposable inoculator swabs (20x50); Catalog No.3323 (100x1).
- **Streakerz™**: Catalog No.3025- Sterile disposable wooden agar plate streakers (10x100); Catalog No.3026 (50x20).
- **Transfer Pipets**: Catalog No.3019- Sterile disposable 9 inch transfer pipets.
- **Reservoirs**: Catalog No.3102- Sterile disposable reservoirs.
- **Multichannel Pipettes:** Catalog No.3711- 8 channel electronic pipettor.
- **Pipet Tips:** Catalog No. 3201- Sterile racked pipet tips for Ovation multichannel pipettor; Catalog No. 3001- Matrix multichannel pipettor tips.
- **Turbidimeter**: Catalog No.3531- 110 volt model, Catalog No.3532 -220 volt model, Catalog No.3585 - 240 volt model.
- **Turbidity Standards**: Catalog No.3441 - 85% T; Catalog No.3440 - 65% T.

Determine Appropriate Protocol to Use (Inoculating Fluid and Cell Density)

- All protocols are performed in the same manner, the only difference being the choice of inoculating fluid (IF) and cell density for inoculation.
- **Protocol A** is used for the vast majority of species.
- **Protocol B** is used for a small number of strongly reducing species and capsulated species (primarily some strains of *Aeromonas, Vibrio,* and spore-forming Gram-positive rods). These species will give a false-positive result in the A-1 well with Protocol A. If this occurs, simply repeat the test using Protocol B.
- **Protocol C1** is used for slow growing bacteria that typically form pinpoint-sized colonies (less than 1 mm in diameter) on BUG+B Agar in 24 hours of growth (see example in **Figure 2.d.**). These are primarily microaerophilic and capnophilic Gram-positive cocci and tiny rods. See Table 1. below for a list.
- **Protocol C2** is used for fastidious, capnophilic, and very oxygen-sensitive bacteria that grow very slowly or not at all on BUG+B Agar. For example, it is used for fastidious Gram-negative species that would most likely be encountered from respiratory tract specimens after cultivation on Chocolate Agar with 6.5% CO_2. Some very oxygen-sensitive Gram-positive bacteria also require the higher inoculation density of Protocol C2. See Table 1. below for a list.
- If unsure of the appropriate test protocol, use protocol A. If the result fails to yield an identification because of a false-positive A-1 well, then use Protocol B. If the result fails because of insufficient positive carbon source reactions, then try, in succession, Protocols C1 and C2.

Table 1. Test Protocols

Protocol	IF	Cell Density	Species
A	A	90-98% T	**Nearly all** – this is the default protocol
B	B	90-98% T	**Strongly reducing and capsule producing GN** (e.g., some *Aeromonas, Vibrio*) **and GP** (e.g., some *Bacillus, Aneurinibacillus, Brevibacillus, Lysinibacillus, Paenibacillus,* and *Virgibacillus*)
C1	C	90-98% T	**Microaerophilic, capnophilic GP** (e.g., *Dolosicoccus, Dolosigranulum, Eremococcus, Gemella, Globicatella, Helcococcus, Ignavigranum, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Weissella,* and some *Aerococcus, Arcanobacterium, Corynebacterium* and *Enterococcus sp.*)
C2	C	62-68% T	**Fastidious, capnophilic, oxygen sensitive GN** (e.g., *Actinobacillus, Aggregatibacter, Alysiella, Avibacterium, Bergeriella, Bordetella, Capnocytophaga, Cardiobacterium, CDC Group DF-3, CDC Group EF-4, Conchiformibius, Dysgonomonas, Eikenella, Francisella, Gallibacterium, Gardnerella, Haemophilus, Histophilus, Kingella, Methylobacterium, Moraxella, Neisseria, Oligella, Ornithobacterium, Pasteurella, Simonsiella, Suttonella,* and *Taylorella*) **and GP** (*Actinomyces, Aerococcus, Alloiococcus, Arcanobacterium, Carnobacterium, Corynebacterium, Erysipelothrix, Granulicatella, Lactobacillus, Pediococcus,* and *Tetragenococcus*)

TEST PROCEDURE

Preparation

- Before starting, prewarm MicroPlates and IF to room temperature and review the entire protocol, including precautions.

Step 1. Culture Organism on Biolog Recommended Agar Media

- **Isolate a pure culture** on Biolog recommended agar media (**BUG+B** or **Chocolate Agar)** and incubate at 33° C. Some species may require special culture conditions, for example either lower or higher temperature (26° - 37° C.) and elevated CO2 (6.5% - 10%).
- **Use of alternative media should be validated**. For laboratories that need to use agar media without blood, we recommend using **BUG Agar**. However, some species will grow extremely slowly or not at all if blood is omitted, for example the genera listed for Protocols C1 and C2 in Table 1. **R2A Agar** and Tryptic Soy Agar without or with blood (**TSA, TSA+B**) can be substituted, but they will not culture as wide a range of bacteria as BUG+B. Furthermore, their recipes and performance characteristics from different vendors may vary.
- **The cells must be freshly grown** since many strains lose viability and metabolic vigor in stationary phase. The recommended incubation period for most organisms is 4-24 hours. Spore-forming gram-positive bacteria (Bacillus and related genera) should be grown for less than 16 hours to help minimize sporulation.
- **If insufficient growth is obtained** to inoculate the panel, restreak heavily (as a lawn) onto one or more agar plates. Incubate for 4-48 hours. This should give enough growth to inoculate the panel.

Step 2. Prepare Inoculum

- **Check the calibration of the turbidimeter** periodically. Use an appropriate turbidity standard (85% T or 65% T) and follow instructions in the turbidimeter manual to verify that the turbidimeter is calibrated and operating properly.
- **Blank the turbidimeter** with a clean tube (wiped clean of dirt and fingerprints) containing uninoculated IF. Because the tubes used are not optically uniform, they should be blanked individually. Set the 100% transmittance adjustment knob so that the meter reads 100%.
- **Prepare the inoculum at the desired turbidity.** The target **cell density should be in the range of 90-98%T for Protocols A, B, and C1**. **Protocol C2 requires a higher cell density of 62-68%T** for species that are sensitive to oxygen. **Use a cotton-tipped Inoculatorz swab** to pick up a 3 mm diameter area of cell growth from the surface of the agar plate. As shown in **Figure 2.a.**, grasp the swab at its tip and, holding the swab vertically, touch it to the cell growth. **Figure 2.b., c., and d.** show examples of fast, medium, and slow growing bacteria, and the yellow circle indicates where to touch the end of the cotton swab. For fast growing bacteria, touch a single colony, for medium growing bacteria, touch a cluster of colonies, and for slow growing bacteria touch the first area of confluent growth. Release the bacteria into the IF by rubbing the swab tip against the bottom of the tube containing IF as shown in **Figure 2.e.** Crush any cell clumps against the tube wall or remove them from the IF by catching them on the swab. Stir the IF with the swab to obtain a uniform cell suspension and read it in the turbidimeter, as shown in **Figure 2.f.** If the cell density is too low, add more cells. If the cell density is too high, add more IF.

  For extremely clumpy bacteria that cannot be dispersed directly, use the following procedure. First prepare a dense suspension in 2 ml of IF as follows. **Use a sterile wooden Streakerz stick** to remove a clump of cell mass from the agar surface without gouging the agar. If the bacteria are extremely dry and embedded in the agar, use the edge of a **sterile glass microscope slide** to gently scrape a mass of cells onto the glass slide, again, without gouging the agar. The cells can then be scraped off the glass slide with a sterile Streakerz stick. Then use the Streakerz stick to deposit the cell mass onto the inner wall of a **dry tube**. Use the Streakerz stick to crush, break up, and spread the clumps of cells against and along the inner wall of the tube. Then add 2 ml of IF, and gradually slide the dispersed cells into the IF. The resulting cell suspension will be a mixture of suspended cells and residual clumps. Stand the tube in a rack for about 5 minutes and allow the clumps to settle to the bottom. Use a small pipet and transfer the suspended cells at the top into a fresh tube of IF to achieve the target cell density.

Step 3. Inoculate MicroPlate

- **Pour** the cell suspension into the multichannel pipet reservoir.
- **Fasten** 8 sterile tips securely onto the 8-Channel Repeating Pipettor and fill the tips by drawing up the cell suspension from the reservoir.
- **Fill all wells with precisely 100 µl** as shown in **Figure 2.g.** Be careful not to carry over chemicals or splash from one well into another. The inoculating fluid will form a soft gel shortly after inoculation.
- **Cover the MicroPlate** with its lid and eject the pipettor tips.

Step 4. Incubate MicroPlate

- **Place the MicroPlate** into the OmniLog incubator/reader as shown in **Figure 2.h.**, or into an incubator, for 3 to 36 hours. Incubate at 33° C., or use incubation conditions that were found to be optimal for the bacterium in Step 1.

RESULTS

Reading and Interpretation of Results

- **Read MicroPlates** using Biolog's Microbial Identification Systems software (e.g.OmniLog® Data Collection). Refer to the User Guide for instructions.
- **Biolog's Microbial Identification Systems Software performs all reading and interpretation of results.**
- The color densities in wells of the **carbon source utilization assays** in columns 1-9 are **referenced against the negative control well, A-1**. All wells visually resembling the A-1 well should be scored as "negative" (-) and all wells with a noticeable purple color (greater than well A-1) should be scored as "positive" (+). Wells with extremely faint color, or with small purple flecks or clumps are best scored as "borderline" (\). Most species give dark, clearly discernible "positive" reactions. However, it is normal for the "positive" reactions of certain genera to be light or faint purple.
- The color densities in wells of the **chemical sensitivity assays** in columns 10-12 are **referenced against the positive control well, A-10**. All wells showing significant sensitivity to the inhibitory chemical, with less than half the color of the A-10 well are considered "negative" (-) for growth. All other wells showing normal or near normal purple color (similar to well A-10) are considered "positive" (+). If there is uncertainty about the interpretation, it is best to score the well as "borderline" (\).
- **"False positive" color** is defined as purple color forming in the negative control well (A-1) and in other "negative" wells. This is seen with only a few species such as from the genera ***Aeromonas, Vibrio,*** and ***Bacillus***. If such a result occurs, the cells are simply retested with Protocol B and IF-B.
- **See Biolog's Microbial Identification Systems software User Guide for further assistance in interpreting identification results.**

Precautions

To obtain accurate and reproducible results, **the recommendations below must be followed**.

- **Read** the "Instructions for Use" prior to using the GEN III MicroPlate and follow the procedures.
- **Pure cultures must be used** to obtain identifications. The system is not designed to identify individual bacterial strains from within mixed cultures. The most common problem in identification is that microbiologists are not aware that they have a mixed culture. Streaking for isolated colonies may not be sufficient because isolated colonies can arise from a clump of cells as well as a single cell. Bacteria have sticky surfaces and they tightly adhere to other bacteria. **This is particularly a problem with mucoid bacteria, fresh environmental isolates, and staphylococci.** First, examine cultures with care using a dissecting microscope or some colony magnifying lens, to make sure that only one colony morphology is present in the culture. **If no species identification is obtained, you may still have a mixed culture.** Restreak the cells onto a multi-chromogenic agar medium and let the original agar plate and the chromogenic agar plate sit at room temperature for 3 or 4 days. Examine both plates carefully, looking for the outgrowth of "bumps" or non-uniform growth in the areas of confluent growth. On the chromogenic agar plate, look for more than one color. If necessary, reisolate the colony types that are present and perform the identification assay a second time.

- **Culture media and repeated subculturing** may affect the results. Strains may produce different phenotypic patterns depending upon how they are cultured prior to inoculation.
- **Sterile** components and aseptic techniques must be used in set-up procedures. Contamination will affect results.
- **Disposable glassware** should be used to handle all cell suspensions and solutions. Glassware that has been washed may contain trace amounts of soap or detergent that will affect results.
- **Prewarm** the IF and the MicroPlates to room temperature before use. Some species (e.g., *Neisseria sp.*) are very sensitive to cold shocks.
- **Check the calibration of** your turbidimeter carefully and **always prepare your inoculum within the specified density range.**
- **Biolog's chemistry** contains components that are sensitive to temperature and light. Store the inoculating fluids in the dark with refrigeration. Brown or yellow wells in the GEN III MicroPlate indicate deterioration of the chemistry.
- **Always keep in mind** that you are testing the metabolic properties of **live cells**. Some species can lose their metabolic vigor when subjected to stresses (e.g., temperature, pH, and osmolarity) for even a few minutes. To get the best performance possible from these MicroPlates, be aware that the cells are alive and take care in how you handle them.

Trouble Shooting

If all wells in columns 1-9 are positive, make sure that:

- You are using a microorganism that is appropriate for the GEN III MicroPlate. If the bacterium is a strongly reducing or capsulated species causing false positive color in the A-1 well, repeat the test using Protocol B and IF-B.
- You are not carrying over any nutrients from the agar growth medium into the inoculating fluid.
- Your inoculum is free of all clumps.
- Your inoculum density is not excessive – check the calibration of your turbidimeter.
- The A-1 well is not under-filled. It is used as a reference well by Biolog's Microbial Identification Systems software.

If all wells in columns 1-9 are negative, make sure that:

- You are using a microorganism that is appropriate for the GEN III MicroPlate. Oligotrophic species or extremely slow growing or oxygen sensitive bacteria, for example, may give all negative wells.
- Your cells are freshly grown and you have used the recommended agar culture medium.
- Your incubation temperature and atmosphere are correct for the organism that is being tested.
- The inoculating fluid was stored correctly and was prewarmed prior to use.
- You are handling the cells with all disposable hardware (soap residues are toxic).
- Your inoculum density is sufficient – check the calibration of your turbidimeter.
- The A-1 well is not over-filled. It is used as a reference well by Biolog's Microbial Identification Systems software.

Performance Characteristics

The GEN III MicroPlate performance characteristics have been determined by establishing a database using a large collection of microorganisms from diverse sources. The database is designed to give identifications of all species in the database, in accordance with current standards of classical identification methods and current taxonomic nomenclature. **To obtain accurate and reproducible results, all procedures and recommendations in these Instructions for Use must be followed precisely.**

Limitations

The GEN III MicroPlate is designed to identify pure cultures of Gram-negative and Gram-positive bacteria. The panel will only identify members of the species in the current database. Other species will usually be reported out with the message "no identification." Atypical strains may also yield a low similarity index and therefore will be reported out as "no identification." **This product is not for human in vitro diagnostic use.** Some bacterial species are reportable to government and public health agencies in certain circumstances. For any isolate that is identified as ***Salmonella* or *Shigella or E. coli O157:H7,*** we recommend confirmation by serology. ***Neisseria gonorrhoeae*** identifications should also be confirmed. Appropriate caution and confirmation should be used for isolates suspected of being **Dangerous Pathogens**.

Quality Control
Biolog MicroPlates are tested and meet internal quality control standards before being released for sale. However, some laboratories may desire or may be required to perform independent quality control checks on each manufacturing lot.

To test the performance of the GEN III MicroPlate use the 2 Gram-negative and 2 Gram-positive strains specified below using Protocol A. These are available from Biolog as a set (Biolog Catalog No.8050).

1. *Escherichia coli*	ATCC 11775
2. *Paenibacillus polymyxa*	ATCC 842
3. *Staphylococcus epidermidis*	ATCC 12228
4. *Stenotrophomonas maltophilia*	ATCC 13637

Inoculate each bacterium following the TEST PROCEDURE as specified. When lyophilized or frozen cultures are used, they should be **subcultured at least twice** before being tested.

Read the panels after appropriate incubation. The resulting identification should correctly correspond to the identity of the quality control strain.

If the identification does not match, review the test procedures and check the purity of your culture. Repeat the test.

Technical Assistance
For help or to **report problems** with this product contact Biolog Technical Service either by phone (510-785-2564) by fax (510-782-4639) or by email (tech@biolog.com) during business hours (7:30 A.M. to 5 P.M. Pacific Standard Time), or contact the Biolog Distribution Partner in your area.

General information, Certificates of Analysis and MSDS can now be found at www.biolog.com.

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B. Biolog EcoPlate™ Instructions for Use

BIOLOG **Microbial Community Analysis**

EcoPlate™

A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine	A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine	A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine
B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine	B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine	B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine
C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine	C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine	C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine
D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine	D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine	D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine
E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine	E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine	E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine
F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid	F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid	F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid
G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine	G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine	G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine
H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine	H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine	H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine

FIGURE 1. Carbon Sources in EcoPlate

INTRODUCTION

Microbial communities provide useful information about environmental change. Microorganisms are present in virtually all environments and are typically the first organisms to react to chemical and physical changes in the environment. Because they are near the bottom of the food chain, changes in microbial communities are often a precursor to changes in the health and viability of the environment as a whole.

The Biolog EcoPlate™ (Figure 1) was created specifically for community analysis and microbial ecological studies. It was originally designed at the request of a group of microbial ecologists that had been using the Biolog GN MicroPlate™, but wanted a panel that provided replicate sets of tests[1].

Community analysis using the Biolog MicroPlates was originally described in 1991 by J. Garland and A. Mills[2]. Researchers found that by inoculating Biolog GN MicroPlates with a mixed culture of microorganisms and measuring the community fingerprint over time, they could ascertain characteristics about that community of microbes. This approach, called community–level physiological profiling, has been demonstrated to be effective at distinguishing spatial and temporal changes in microbial communities. In applied ecological research, the MicroPlates are used as both an assay of the stability of a normal population and to detect and assess changes following the onset of an environmental variable.

Studies have been done in diverse applications of microbial ecology and have demonstrated the fundamental utility of Biolog MicroPlates in detecting population changes in soil, water, wastewater, activated sludge, compost, and industrial waste. The utility of the information has been documented in over 500 publications using Biolog technology to analyze microbial communities. A bibliography of publications is posted on the Biolog website at www.biolog.com/mID_section_4.html.

ECOPLATE

The Biolog EcoPlate contains 31 of the most useful carbon sources for soil community analysis. These 31 carbon sources are repeated 3 times to give the scientist more replicates of the data. Communities of organisms will give a characteristic reaction pattern called a metabolic fingerprint. These fingerprint reaction patterns rapidly and easily provide a vast amount of information from a single Biolog MicroPlate.

The community reaction patterns are typically analyzed at defined time intervals over 2 to 5 days. The changes in the pattern are compared and analyzed using statistical analysis software. The most popular method of analysis of the data is via Principle Components Analysis (PCA) of average well color development (AWCD) data, but alternative methods may also offer advantages[3–8]. The changes observed in the fingerprint pattern provide useful data about the microbial population changes over time.

Biolog MicroPlates have been compared to other methods, such as phospholipid fatty-acid analysis, for monitoring community and ecological changes. The MicroPlates were found to be more sensitive to changes in the environment[9]. Biolog MicroPlates were also indicated as more sensitive to changes in major determinants such as temperature and water.

Similar analyses have been performed using the Biolog GN and GN2 MicroPlates. For certain applications the GN2 MicroPlates may be preferable to the EcoPlate. The individual application will dictate which MicroPlate is best suited for the community or ecological analysis.

TYPICAL PROCEDURE[3]

***STEP 1*:** Environmental samples are inoculated directly into Biolog MicroPlates either as aqueous samples or after suspension (soil, sludge, sediment, etc…).

***STEP 2*:** The Biolog MicroPlates are incubated and analyzed at defined time intervals.

***STEP 3*:** The community-level physiological profile is assessed for key characteristics:

- Pattern development (similarity)
- Rate of color change in each well
- Richness of well response (diversity)

Formation of purple color occurs when the microbes can utilize the carbon source and begin to respire. The respiration of the cells in the community reduces a tetrazolium dye that is included with the carbon source.

The reaction patterns are most effectively analyzed with a microplate reader, using the MicroStation System. However, any good microplate reader can be used to provide optical density (OD_{590}) values.

Statistical analysis of the data is typically performed using standard software packages. Some researchers have found that PCA provides greater resolution than other methods of statistical analysis[10].

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[10] Defining soil quality in terms of microbial community structure. M. Firestone, T. Balser, D. Herman, Annual Reports of Research Projects, UC Berkeley, 1997

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Part# 00A 012, Rev. B, July 07

C. Bio Plug Installation Instructions

Instructions For The Installation and Operation of Demonstration and Test Bio Plugs
July 2007

1.0 Configuration

A. Demonstration and Test Bio Plugs

1. Bio plugs for demonstration and test purposes may new plugs installed within known formations or renovation of existing monitoring wells.
2. The location of screens shall be in the zone of contamination above confining layers. Multiple screens may be used with multiple contaminated zones.
3. Water is to be prohibited from entering any zone above or below the contaminated zone if the permeability of the other zones are greater than the zone of contamination.
4. Conversion of monitoring wells may be dealing with contaminated zones above the screens. This situation should be taken into account when evaluating the test results. The contaminants above the screened area will move into the screened zone and the test results may not indicate a steady drop of the contaminant concentration in down stream monitoring wells.

B. Bio Plugs

1. The bio plugs are to be constructed from schedule 80 PVC or HDPE, SDR 17 pipe as determined by the site conditions and contaminants.
2. The bio plugs are typically 4 inches in diameter. They may be installed as direct bore or inserted into existing treatment wells or monitoring wells. The screened portion of the bio plug should closely match the screened portion of the treatment well or monitoring well if used as inserts.
3. A single air line is to be installed in the bio plug. The air line shall be installed to the bottom of the bio plug with a fine bubble diffuser installed on the end.
4. A single nutrient line is to be installed in the bio plug. The nutrient line shall be installed to the bottom of the bio plug media and may have one opening at the bottom or multiple small openings within the depth of the media. The location and size of the openings shall be discussed with ABS prior to installation of the nutrient line.
5. The air and nutrient connections to the bio plug shall be isolated with ball valves located within the head box of the bio plug. The valves are to be used to throttle the feed rate of air and nutrients to match the permeability of the contaminated layer that is being treated.
6. All air and nutrient piping and valves within the head box and bio plugs shall be 1/4 inch ID unless otherwise required to provide high rates of air and nutrient flow.
7. The screened portion of the bio plug shall be “well screen” with 0.125 inch slot openings.
8. The bio plugs are to be put together in 10 foot sections or less. HDPE connections are to be screwed and PVC sections are to be glued coupled or screwed, dependent on the circumstance of installation.

9. Head pieces are to be provided by ABS and shall be made to be removable from the bio plug for maintenance of the interior tubing and fittings.

10. A head box is to be provided for each bio plug, either flush mounted with the top of the ground or traffic area or above ground boxes for bio plugs that are extended above the ground in non traffic areas.

2.0 Installation:

A. Drilling and Packing

1. The holes for the bio plugs are to be drilled using a hollow stem auger. The minimum size of the hollow stem shall be 10 inches for 4 inch ID and smaller bio plugs. For larger diameter bio plugs, the hollow stem shall be two (2) times the inside diameter of the bio plug.

2. The bio plugs are to be inserted in the hollow stem and the area around the bio plug screens shall be packed with washed pea gravel with a minimum diameter of 0.165 inches. The gravel pack shall extend 6 inches +/- above and below the top and bottom of the screen.

3. A bentonite grout shall be placed above and below the screen packing to isolate the soil layer to be treated.

4. Drill cuttings with contaminant concentrations greater than the local REPO standards are to be placed in a roll off box and properly disposed of off site in a state approved landfill.

5. The remainder of the packing above the screens may be pea gravel or sand. The top 18 inches of the packing shall be cement grout to seal off the packing to prevent air and water from moving upward from the treatment layer. Water flood or vibrate the loose packing to consolidate it and minimize settling after the bio plug is in operation.

6. The top 12 to 18 inches of the packing shall be cement grout to further seal the packing from the surface.

B. Bio Plugs

1. Bio plugs are to be assembled in the field and installed in the hollow stem auger after the hole has been drilled. The pipe shall be assembled in 10 foot sections or shorter depending on the job requirements.

2. The bio plugs are to be assembled using screwed threaded HDPE or glued coupled schedule 80 PVC. The connections are to be tight before inserting into the hollow stem. Glued connections are to be set and strong enough to withstand the vertical weight of the piping while inserting into the hollow stem.

3. The pipe is to be positioned in the center of the hollow stem and held in place while the bentonite grout and packing materials are placed.

4. After placing and packing the bio plug, a temporary cap is to be put over the top of the bio plug to prevent dirt and debris from entering the bio plug.

5. Before placing media in the bio plug, insert the air and nutrient tubing and hold in place at the top of the bio plug. The air line shall be placed to the bottom of the bio plug and the nutrient line shall be placed to the bottom of the media.

5. After placing the air and nutrient lines, the media is to be deposited in the pipe to completely fill the screened portion of the bio plug. The remainder of the bio plug is to remain free of media.

C. Head Boxes

1. For flush mounted bio plugs, install a head box suitable for the installation area. In traffic area, install a traffic rated box and in non traffic areas such as lawns or fields a non traffic rated box may be used.
2. Install the head box having enough room to allow for tubing connections and installation of ¼ inch throttling valves on the air and nutrient lines.
3. Place 3 to 5 inches of washed stone under the box. Firmly support the box on this stone making sure that the top of the box is level and flush with the top of the ground. No part of the box should protrude above the ground far enough to be hit by mowers or damaged by traffic.
4. Side openings shall be provided in the head box to allow the entrance of conduit with air and nutrient tubing.

3.0 Nutrient Feed

A. General

1. Nutrients are fed to the bio plug to enrich the water surrounding the media for the purpose of increasing the microbial population concentration used to degrade the contaminants in the ground water and surrounding soil.

B. Nutrient Mix

1. The nutrients shall consist of nitrogen in the form of ammonia or urea, phosphorous in the form of phosphate or P_2O_2 and carbon in the form of acetate or low concentrations of the contaminant being treated. The general ratio of nutrients is 100 parts Carbon: 10 parts N: 1 part P.
2. The nutrients are mixed with water and pumped into the bio plugs. The amount of nutrient mix pumped to each plug depends on the contaminant concentrations and on the permeability of the contaminated layer of soil. The more porous the soil, the higher the rate of nutrient feed.
3. The nutrients shall be supplied in accordance with the following:

Standard Liquid Nutrient Mix:

Nutrient	Supplier	Product Name	Amount
Carbon	Rapid Drilling	50% Potassium Acetate	1.00 gal.
Nitrogen	PCS Sales	Uran 32	.10 gal
Phosphorous	PCS Sales	Ammonium Phosphate	.03 gal
		Total	1.13 gal

Rapid Drilling Products, LLC, 910 Harding Street, Lafayette, LA 70503, Phone 337-291-2778, PCS Sales1101 Skokie Blvd, Northbrook, IL 60062, Phone 800-241-6908

4. The amount of nutrient solution that is to be fed to the bio plugs is dependent on the concentration of contaminant to be treated and the amount of media used in the bio plugs. The standard feed rate per bio plug is 0.01 gallons per day of the undiluted nutrient mix. The nutrient mix is to

be diluted as needed to match the capacity of the nutrient feed pump and the permeability of the contaminated soil layer.

5. The range of enriched (diluted) nutrient feed will normally vary from 0.2 to 0.5 gallons per hour per bio plug.

4.0 Air Feed

A. General

1. Air is to be fed to the bio plugs for the purpose of keeping the water column surrounding the media and the zones immediately surrounding the bio plug aerobic.

B. Feed Rate

1. Air is to be fed to the bio plugs at a rate sufficient to maintain aerobic conditions but low enough to prevent stripping volatile compounds or creating excessive turbulence in the media column.

2. The air pressure required for the bio plug shall be sufficient to overcome the pressure of the maximum level of the water column in the bio plug plus head losses of 5 psig.

3. The air shall be fed continuously at rates of 2 to 5 standard cubic feet per hour (scfh). The rate of air feed may vary within the treatment area based on the depth and amount of media used in the plugs.

VITA

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